

RBL-2H3 cells are an imprecise model for mast cell mediator release

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Abstract

Objective The cell line, RBL-2H3, has been widely used as a mast cell model though much of the data is contradictory. The aim of this study is to assess the RBL-2H3 cell line as an in vitro model for degranulation studies.

Methods RBL-2H3 cells were stimulated with either dinitrophenylated-IgE, calcium ionophore A23187, compound 48/80, mast cell degranulating peptide or lipopolysaccharide and mediator (histamine, β -hexosaminidase, interleukin-13 and TNF- α) release was analysed. Toll-like receptors (TLR) mRNA expression in RBL-2H3 cells and rat peritoneal mast cells (RPMC) were compared by RT-PCR. TLR4 and CD14 surface proteins in RBL-2H3 were analysed by FACS.

Results Mediator release was dependant on media composition and degranulation was stimulated by IgE and A23187. Degranulation was inhibited by quercetin, but not by cromoglycate or ketotifen. Transcripts encoding TLR3-5 and 6 were detected in RBL-2H3 cells whereas TLR1-6 and TLR8 were clearly seen in RPMC. While proteins were detected for TLR4 in RBL-2H3 cells, CD14 was largely absent.

Conclusions While RBL-2H3 cells may be useful as a model for mast cell IgE-mediated degranulation, other aspects may not be representative and they may share similarities with basophils rather than with other histamine-releasing cell types.

Keywords RBL-2H3 · Mast cell · Toll-like receptors

Introduction

Mast cells play a crucial role in the development of allergic diseases and inflammatory processes such as asthma, mastocytosis, inflammatory arthritis and autoimmune diseases [1]. In addition, it has been suggested that mast cells might also be involved in the adaptative and innate immune response to pathogens via Toll-like receptors (TLR) [2]. Mast cells degranulate in response to peptidoglycan challenge through a TLR2-dependent pathway and are able to release newly synthesised cytokines [e.g., interleukin (IL)-13, IL-4 and TNF- α] after stimulation of TLR4 by lipopolysaccharide (LPS) [3].

The existence of mast cell subclasses is widely accepted, with mast cells in connective tissues demonstrating different staining properties, proteoglycans and histamine content from mast cells in the gut or lung mucosa [4].

Inflammation research is often hampered by difficulties originating from the isolation and primary culture of degranulating cells. For example, mast cells obtained from peripheral blood progenitors suffer from the disadvantage of long and involved protocols for culture and differentiation [5]. Peritoneal mast cells, on the other hand, can be easily obtained by peritoneal lavage of small animals (e.g., rats). However, they need to be purified, which can impede their ability to react to stimuli [6]. In addition, it is impossible to maintain them in primary culture over prolonged periods of time [7].

The continuous rat cell line, RBL-2H3, was cloned by the limited dilution technique from leukaemia cells isolated from rats after treatment with the chemical carcinogen,

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β -chloroethylamine [8]. RBL-2H3 cells have been extensively used for studying IgE-Fc ϵ RI interactions [9], signalling pathways for degranulation [10] and to test novel mast cell stabilisers [11]. Therefore, RBL-2H3 cells can be considered a useful tool for in vitro work, as a great number of monoclonal cells can be rapidly obtained by simple cell culture techniques. Despite the large amount of information available on RBL-2H3 cells, a lack of consistency exists between findings from different groups. One aim of this paper was therefore to investigate factors that influence the experimental behaviour of RBL-2H3 cells and to optimise the conditions for the most commonly used assay performed with this cell line.

Materials and methods

Cell culture

RBL-2H3 were purchased from LGC Promochem (Middlesex, UK), from the American Type Culture Collection (ATCC), ATCC cat #CRL-2256 (RBL-2H3). RBL-2H3 cells were cultured in α -MEM (GIBCO, Paisley, UK) supplemented with 10% foetal bovine serum (FBS, Biosera, Ringmer, UK) and 100 U ml⁻¹ penicillin-streptomycin (GIBCO) at 5% CO₂ and 37°C. Cells were subcultured using Accutase (Sigma-Aldrich Ireland Ltd., Dublin, Ireland) when reaching 80% confluency and were plated at $1.2\text{--}2.8 \times 10^5$ cells/cm² in 24 or 6-well plates for experiments as outlined below.

Rat peritoneal mast cell isolation and purification

Rat peritoneal mast cells (RPMC) were obtained by peritoneal lavage with pre-warmed buffered salt solution (BSS) (137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 0.5 mM CaCl₂, 0.4 mM NaH₂PO₄, 5.6 mM glucose and 10 mM HEPES). Male Wistar rats (200–300 g) were killed in atmosphere saturated with CO₂. Then, BSS (15–20 ml) was injected i.p. and the abdomen was gently massaged for 3 min. The crude cell suspension was aspirated following a mid-line incision and centrifuged for 5 min at 400 $\times g$ at room temperature. Cells were washed three times with BSS. RPMC were purified using a Percoll/BSA density gradient. Briefly, peritoneal exudate was resuspended in 4 ml of BSS and layered onto 10 ml Percoll solution (1.5 ml of 1.54 M NaCl, 27 mM KCl, 3.8 M CaCl₂; 0.75 ml of distilled water; 0.21 ml of 35% BSA solution; 10.5 ml of Percoll; pH 7.2). After centrifugation at 225 $\times g$ for 20 min in a swing out centrifuge, RPMC were separated and resuspended in 10 ml BSS and washed twice.

β -Hexosaminidase and histamine degranulation assays

RBL-2H3 cells were let to adhere for at least 3 h, before the medium was removed and replaced with fresh medium containing either anti DNP-IgE (72.2 ng ml⁻¹) (Sigma, Dublin, Ireland) or mast cell degranulating peptide (MCDP) (10 μ g ml⁻¹), compound 48/80 (10 μ g ml⁻¹) or calcium ionophore A23187 (5 μ g ml⁻¹) (all from Sigma). When anti-DNP IgE was used cells were incubated overnight (16 h), then washed twice with PBS (GIBCO) and subsequently, the releasing medium, Earl's balanced salt solution (EBSS), with 1% bovine serum albumin (BSA, GIBCO), pH 7.4, or balanced salt solution (BSS) (137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 0.5 mM CaCl₂, 0.4 mM NaH₂PO₄, 5.6 mM glucose and 10 mM HEPES) was added, followed by DNP-BSA (150 ng ml⁻¹) (Sigma). Cells were incubated for 30 min at 37°C. Supernatants were analysed (see below).

When MCDP, compound 48/80 or calcium ionophore A23187 were used, supernatants were analysed after 30 min using the following methods: β -hexosaminidase was assayed according to a previously published method [12]. Briefly, 30 μ l of supernatant was transferred into a 96-well plate. Fifty microlitres of substrate solution were added (1.3 mg ml⁻¹ of *p*-nitrophenyl *N*-acetyl-D-glucosaminide in citrate buffer, pH 4.5) and the plate was incubated for 1 h at 37°C. The reaction was stopped by adding 80 μ l of NaOH (0.5 M) to each well and the formed *p*-nitrophenolate was measured spectrophotometrically in a 96-well plate reader (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK) at a wavelength of 405 nm. The absorption was converted into the percentage of total cellular β -hexosaminidase content by comparison with the absorption produced by a Triton $\times$ -100 (Sigma) lysate of the same cells according to the following equation:

$$\text{total enzyme activity\%} = \frac{[(\text{sec reted} - \text{spontaneous})/\text{total content}]}{1} \times 100. \quad (1)$$

The amount of histamine was assayed either using a histamine enzyme-linked immunosorbent assay (ELISA) kit (Labor Diagnostika Nord, Nordhorn, Germany) according to the manufacturer's directions or by using a previously described fluorometric assay [13]. Briefly, histamine reacts with *o*-phthalaldehyde (OPT) to form a fluorophore. To 0.5 ml of supernatant from each well, 0.1 ml of 1 M NaOH and 25 μ l of OPT (1% (w/v) in methanol) were added. The supernatant was then incubated at room temperature for 4 min. The reaction was stopped by addition of 50 μ l of 3 M HCl. Precipitated proteins were removed by centrifugation and 200 μ l aliquots of the supernatant were transferred into 96-well plates. The concentration of the fluorescent product of the reaction

was measured using a FLUOstar OPTIMA microplate reader set at: $\lambda_{\text{ex}} = 360 \text{ nm}$ and $\lambda_{\text{em}} = 450 \text{ nm}$, respectively.

Several studies were performed to obtain a basic understanding of the mediator release from RBL-2H3 cells.

Optimum dose of anti-DNP IgE

In order to determine the optimum dose of anti-DNP IgE, increasing concentrations ($1\text{--}5,000 \text{ ng ml}^{-1}$) were used to stimulate β -hexosaminidase release.

Composition of the releasing medium

Due to differences in salt composition and concentration between releasing media used [12, 14], the effect of two commonly used releasing media (EBSS and BSS; see above), with or without supplementation of BSA (at 0.1%) on the degranulation of RBL-2H3 cells was investigated.

Stimuli and inhibitor studies

Three non-immunological stimuli were applied to trigger RBL-2H3 cell degranulation. We investigated MCDP (a peptide component of bee venom), the polybasic compound 48/80 (both at $10 \mu\text{M}$) and the calcium ionophore A23187 (at $5 \mu\text{g ml}^{-1}$). In addition, three different inhibitory compounds [ketotifen fumarate, disodium cromoglycate (DSCG) and quercetin; all at $10 \mu\text{M}$] were tested as RBL-2H3 cell stabilisers.

Cytokine assay

Mast cells have been reported to respond to components of the outer wall of gram negative bacteria such as LPS, resulting in the production and release cytokines such TNF- α and IL-13. To test the amount of tumour necrosis

factor- α (TNF- α) and interleukin (IL)-13, RBL-2H3 cells were seeded at a density of $0.6\text{--}1.4 \times 10^5 \text{ cells/cm}^2$ in 24-well plates. Cells were incubated for 16 h in the presence of $0.1\text{--}1 \mu\text{g ml}^{-1}$ of LPS. Levels of TNF- α and IL-13 were measured in RBL-2H3 cells' supernatant using ELISA kits (Biosource, Nivelles, Belgium) according to the manufacturer's instructions. The study was performed in triplicate using cells from three different passages.

Polymerase chain reaction (PCR)

Total RNA was isolated from RBL-2H3 and purified RPMC using an RNeasy mini kit (Qiagen, Crawley, UK) according to the manufacturer's instruction. A nitrocellulose spin column was used to avoid heparin contamination of the RNA specimens. It has been postulated that the presence of heparin can inhibit the reverse transcription polymerase chain reaction (RT-PCR), [15]. Total RNA ($4 \mu\text{g}$) was treated with a TURBO DNA-free kit (Ambion, London, UK) to remove contaminating genomic DNA. The RNA preparation was reverse transcribed to cDNA using a cDNA synthesis kit (Bioline, London, UK) following the manufacturer's instruction and amplified using a BioTaq core kit with BioTaq DNA polymerase (Bioline). Primers were designed on *Rattus norvegicus* genes using Primer3 software (<http://fokker.wi.mit.edu/primer3/input.htm>) and synthesised by MWG-Biotech (Ebersberg, Germany). Sequences for PCR primer pairs, annealing temperatures and size of the transcripts for TLRs are shown in Table 1. The PCR reaction contained $4 \mu\text{l}$ of cDNA, $0.4 \mu\text{M}$ of each primer 2 mM MgCl_2 .

The PCR cycling conditions were: 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, annealing temperature for 45 s, 72°C for 45 s and a final cycle at 72°C for 10 min. Ten microlitres of the product were run on a 2% agarose gel (Bioline, London, UK) and visualised by ethidium bromide staining using a ChemiDoc gel documentation

Table 1 Primer sequences for RT-PCR

	Forward primer	Reverse primer	Annealing temperature ($^\circ\text{C}$)	Product size (bp)
TLR1	5'-TACCCTGAACAACGTGGACA-3'	5'-ATCGACAAAGCCCTCAGAGA-3'	58	165
TLR2	5'-GGAGACTCTGGAAGCAGGTG-3'	5'-CGCCTAAGAGCAGGATCAAC-3'	58	245
TLR3	5'-AGCCTTCAACGACTGATGCT-3'	5'-GTTGGCAATGTTGTTGTTGC-3'	58	120
TLR4	5'-TGCTCAGACATGGCAGTTTC-3'	5'-TCAAGGCTTTTCCATCCAAC-3'	58	206
TLR5	5'-GCCAGAGCCAGATTGAAGTC-3'	5'-TGTGAATCTCGTTGGCAGAG-3'	58	168
TLR6	5'-GTCTCCCCACTTCATCCAGA-3'	5'-AGAGCCCTCAGCTTGTGGTA-3'	56	156
TLR8	5'-CTGTGGAATGCAAATGATGG-3'	5'-TCATTTCTCCCCAAGTCCAG-3'	62	230
TLR9	5'-TCAACAAGAACACGCTCAGG-3'	5'-GAGAGCTGGGGTGAGACTTG-3'	62	176
TLR10	5'-TGCAGGAGCTGAACATGAAC-3'	5'-ATTGGACAGGTCCACAAAGC-3'	60	235
β -Actin	5'-GTCGTACCACTGGCATTGTG-3'	5'-CTCTCAGCTGTGGTGGTGAA-3'	59	181

system (Bio-Rad, Hemel Hempstead, UK). RNA samples from rat lung were included as positive control. β -Actin was amplified as internal control. Gels were calibrated using a Quick-Load[®] 100 bp DNA Ladder [New England Biolabs (UK), Hitchin, UK].

TLR4 and CD14 detection by flow cytometry

Indirect staining for cell surface TLR4 and CD14 protein was obtained with unlabelled polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), followed with ALEXA Fluor-conjugated IgG antibody (Biosciences, Dublin, Ireland). One million cells were suspended in 100 μ l of PBS charged with 0.1% of BSA. Subsequently cells were incubated with the primary antibody (20 μ g ml^{-1}) for 15 min and fixed with reagent A from Fix and Perm kit (Biosciences, Dublin, Ireland) for additional 15 min. Cells were then washed three times in 3 ml of PBS supplemented with 0.1% NaN_3 and 5% FBS. After washing cells were resuspended in 300 μ l of PBS/1% BSA and incubated in the dark for 15 min with the secondary antibody. Cells were washed three times as previously described, samples were transferred to a 96 well plate and analysed by flow cytometry for single-colour staining using a BD FACS Array (BD Biosciences, Oxford, UK).

Statistical analysis

The results are presented as mean $\pm$ SEM unless otherwise specified. Statistical analysis of several groups of data was performed by one way analysis of variance, ANOVA, followed by Bonferroni's test. Statistical

analysis between two groups was by means of the unpaired, two-tailed Student's *t* test. *P* values <0.05 were considered significant.

Animals were killed according to guidelines laid down by the working party report [Laboratory Animals (1996) 30, 293–316, Laboratory Animals (1997) 31, 1–32], on Directive 86/609/EEC (No. L 358, ISSN 0378-6978), which is endorsed by the Bioresources Ethical Review Committee of the University.

Results

β -Hexosaminidase and histamine degranulation assays

RBL-2H3 cells were sensitised with increasing concentrations of anti-DNP IgE (1–5,000 ng ml^{-1}) and triggered with constant concentrations of allergen (150 ng ml^{-1}). Maximum release of β -hexosaminidase occurred at a concentration of 72.2 ng ml^{-1} of anti-DNP IgE with a value of $20.32 \pm 0.8\%$ of the total enzyme activity. Thereafter, increasing concentrations of anti-DNP IgE resulted in a progressively reduced release of β -hexosaminidase, with 5,000 ng ml^{-1} of anti-DNP IgE releasing $7.0 \pm 5.5\%$, representing 34.5% of the maximum value at 72.2 ng ml^{-1} anti-DNP IgE. A total amount of $36.6 \pm 6.3 \text{ ng ml}^{-1}$ released histamine was detected by ELISA, while histamine levels using the fluorometric assay were significantly higher at $1,482 \pm 85 \text{ ng ml}^{-1}$. The total amount of histamine per 10^6 cells was calculated to be approximately 0.12 μg .

Figure 1 shows how the degranulation of RBL-2H3 cells was affected by the composition of releasing media.

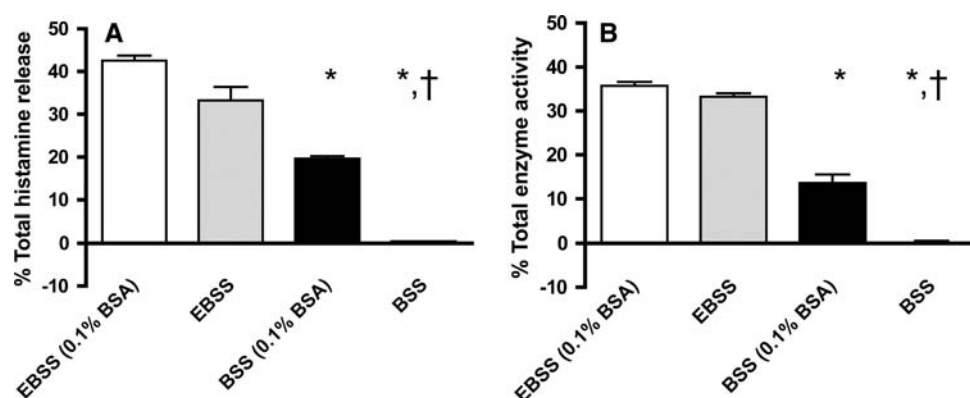


Fig. 1 **a** Effect of the releasing media composition on IgE-induced (78.12 ng/ml) release of β -hexosaminidase from RBL-2H3. *Open square* Earle's balanced salt solutions (EBSS) +0.1% of bovine serum albumin (BSA), *shaded square* EBSS, *filled square* balanced salt solution (BSS) +0.1% BSA. **b** Effect of the releasing media composition on the histamine release from RBL-2H3. *Open square* Earle's balanced salt solutions (EBSS) +0.1% of bovine serum

albumin (BSA), *shaded square* EBSS, *filled square* balanced salt solution (BSS) +0.1% BSA. Each value represents the mean $\pm$ SEM, $n = 3$. The release of β -hexosaminidase and histamine followed the same trend. *Indicates significantly different ($P < 0.05$, $n = 3$) from EBSS (with and without BSA); †indicates significantly different ($P < 0.05$, $n = 3$) from BSS without BSA

The release of β -hexosaminidase (1A) and histamine (1B) followed the same trend. The most extensive degranulation was observed when EBSS (+BSA) was used, while a slightly lower value was obtained with EBSS without the BSA supplement. BSS containing 0.1% of BSA resulted in a significantly lower mediator release and BSS without BSA resulted in a complete inhibition of degranulation.

RBL-2H3 response to various stimuli and mast cell stabilisers

Responses of RBL-2H3 cells to MCDP, compound 48/80 and A23187 as well as the effect of the mast cell stabilisers, DSCG, ketotifen and quercetin is illustrated in Fig. 2. A23187 ($5 \mu\text{g ml}^{-1}$) was used to stimulate release of granular mediators resulting in 50% of total β -hexosaminidase release (Fig. 2a), which was approximately 40% higher than values obtained after IgE sensitisation (at 500 ng ml^{-1}). MCDP and compound 48/80 both failed to produce any response. In our hands, quercetin at $10 \mu\text{M}$ reduced the amount of total enzyme release from 31.2 ± 1.8 to $4.5 \pm 1.0\%$, ($P < 0.01$). Ketotifen and

DSCG did not show any cell membrane stabilising activity in the RBL-2H3 cell line (Fig. 2b).

Cytokine release after stimulation with LPS

Lipopolysaccharide (*Escherichia coli* 0111:B4) at concentrations between 0.1 and $1 \mu\text{g ml}^{-1}$ failed to produce a cytokine response from RBL-2H3 cells. The amount of IL-13 released after stimulation with $1 \mu\text{g ml}^{-1}$ of LPS was $35 \pm 10 \text{ pg ml}^{-1}$ which was not significantly ($P > 0.05$) different from the control ($39 \pm 12 \text{ pg ml}^{-1}$), while there was no TNF- α released ($-1 \pm 2 \text{ pg ml}^{-1}$) (Fig. 3).

TLRs in RBL-2H3 cells and RPMC

Figure 4 shows a representative agarose gel with the PCR products for TLR1 through TLR10 in RBL-2H3 cells cultured for 2 days and freshly isolated and purified RPMC. Transcripts encoding TLR3, 4, 5 and 6 were detected in RBL-2H3 cells while messages for TLR1, 2, 8, 9 and 10 were absent. The TLR3 band, although large and smeared, presents a brightness higher than that of usual smear and it is correctly localised right above 100 bp and it

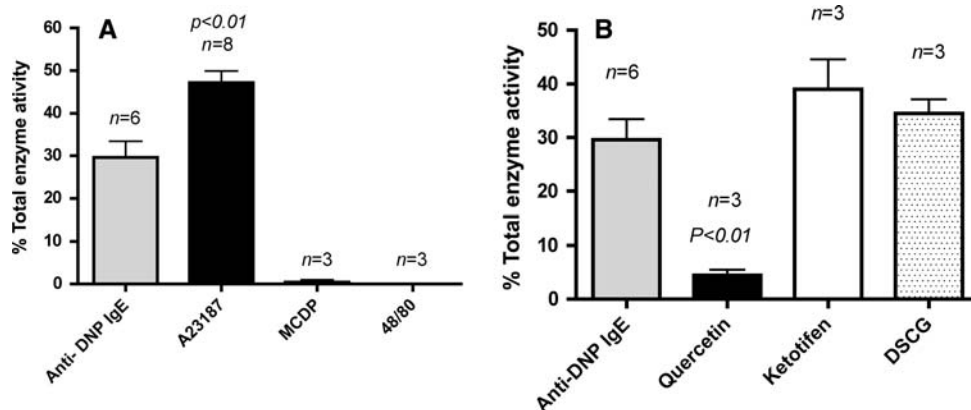
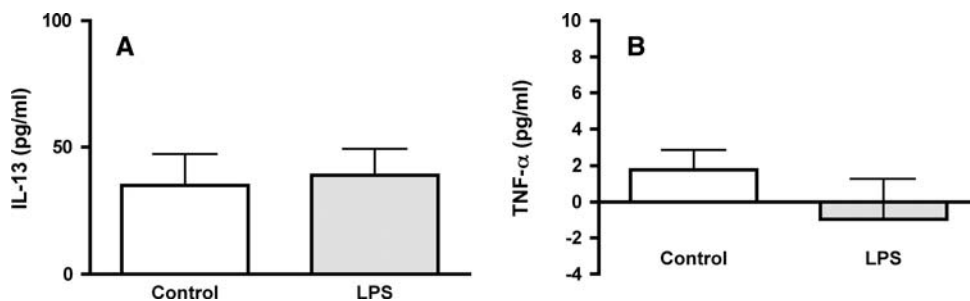


Fig. 2 a Effect of calcium ionophore A23187 ($5 \mu\text{g ml}^{-1}$ filled square), mast cell degranulating peptide (MCDP, $10 \mu\text{g ml}^{-1}$) and compound 48/80 ($10 \mu\text{g ml}^{-1}$) on the β -hexosaminidase release from RBL-2H3 cells. **b** Effect of quercetin ($10 \mu\text{M}$, filled square),

Ketotifen ($10 \mu\text{M}$, open square) and disodium cromoglycate (DSCG, $10 \mu\text{M}$, open square) on the IgE-induced (78.12 ng/ml) release of the granule associated β -hexosaminidase (dotted square) in RBL-2H3 cells

Fig. 3 IL-13 and TNF- α release from RBL-2H3 cells after stimulation with lipopolysaccharide (LPS) ($1 \mu\text{g ml}^{-1}$). Cells were stimulated for 12 h. Data represent the mean $\pm$ SEM, $n = 3$



is definitely separated from the primer dimer region; therefore it is possible to conclude the presence of the mRNA transcript for TLR3. The signals for both TLR2 and TLR8 are not correctly localised. In RPMC, transcripts encoding TLR1, 2, 3, 4, 5, 6 and 8 but not TLR9 were detected. A barely detectable band corresponding to TLR10 suggests the presence of this transcript.

TLR4 and CD14 surface expression

After 3 days in culture, the presence of TLR4 and CD14 in RBL-2H3 cells was investigated by flow cytometry. Two separate one-colour experiments were performed in parallel. The results are summarised in Fig. 5. There was a significant ($P < 0.05$) difference in mean fluorescence intensity (MFI) between control values ($1,260 \pm 555$) and those for TLR4 ($14,206 \pm 5,235$) (Fig. 5a). While TLR4 proteins were detected, confirming the results obtained with the RT-PCR, signals for CD14 were found to be not statistically ($P > 0.05$) different from control values of MFI, being $12,756 \pm 3,164$ and $7,863 \pm 5,253$, respectively (Fig. 5b).

Discussion

The RBL-2H3 cell line has served as an established model system for the study of secretory cells that contain histamine and bear IgE receptors. Degranulation of RBL-2H3 was monitored following the release of two different mediators, granule-associated β -hexosaminidase and histamine. A significant discrepancy was found between the results of the histamine ELISA and the OPT method that might be attributed to the fact that the fluorometric assay is not selective for histamine alone, but can react with other amines to give fluorescent adducts [16]. We therefore decided to use the OPT assay in parallel with the

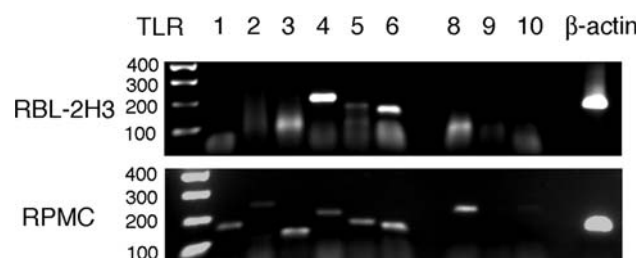


Fig. 4 Representative ($n = 4$) agarose gels from RT-PCR analysis of TLR mRNA expression in RBL-2H3 and RPMC. RT-PCR products for TLR1 through TLR6 and TLR8–TLR10 were analysed. β -Actin was amplified as internal control. The vertical scale on the left of each gel represents markers calibrated in numbers of base pairs, obtained using a 100 bp DNA ladder

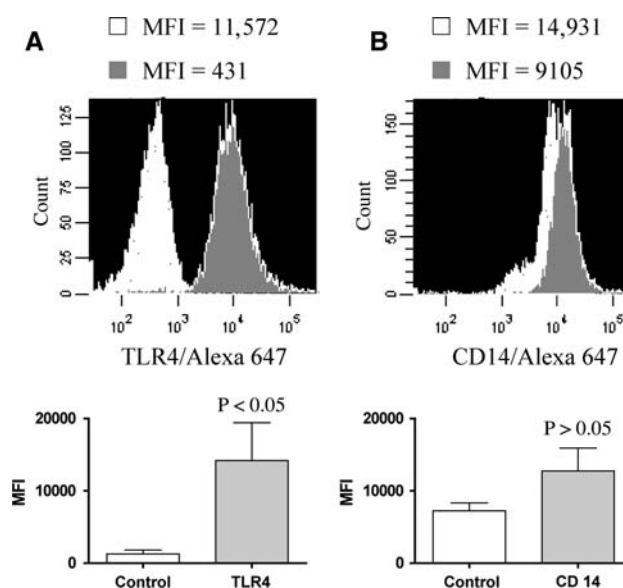


Fig. 5 Presence of TLR4 and CD14 proteins on RBL-2H3 cells. Representative flow cytometry data showing mean fluorescence intensity (MFI) comparing TLR4 (a) and CD14 (b) (shaded) versus negative control (not shaded) in the upper panels. Histogram columns in the lower panels represent the mean values and SEM of MFIs from $n = 5$ (TLR4) and $n = 4$ experiments (CD14), respectively

β -hexosaminidase assay. Results showed that the release of amines closely paralleled the release of enzyme.

The histamine content of RBL-2H3 cells was rather low at approximately $0.12 \mu\text{g}$ per 10^6 cells. Again, this is different from values typically reported for connective tissue mast cells [4] and more within the range reported for mucosal mast cells or basophils.

RBL-2H3 could only be stimulated to release mediators using IgE and the calcium ionophore A23187. Buku et al. [17] reported that MCDP binds to RBL-2H3; however, they did not confirm a resulting degranulation from these cells, although they did report MCDP-mediated degranulation from RPMC. Connective tissue mast cells have been reported to also react to compound 48/80 [4].

Pharmacological studies using mast cell stabilising agents revealed that only quercetin effectively reduced IgE stimulated degranulation in RBL-2H3 cells. Basophils and mucosal mast cells have also been previously shown to be unresponsive to DSCG [18, 19], but these cell types can be stabilised using ketotifen and quercetin [20, 21].

RBL-2H3 cells appear to be a reliable model to study IgE-mediated degranulation of secretory cells. They exhibit a bell-shaped dose-response curve to increasing concentration of anti-DNP IgE [22] and this was confirmed in our study. This phenomenon has been previously reported, not only for the degranulation of mast cells [23] but also basophils [24].

In this study RBL-2H3 cells did not release cytokines after incubation with LPS. This result is inconsistent with

previously published data where others have shown RBL-2H3 releasing up to 180 µg/ml of IL-13 and 60 ng/ml of TNF- α [25]. Our findings are analogous to those of Bieneman and co-workers, who reported that basophils are insensitive to LPS despite the expression of TLR4. They suggested that this behaviour is due to the lack of CD14 [26]. The CD14 co-receptor needs to form a complex with TLR4 in order to allow further signalling [27]. In this study we looked for CD14 expression in RBL-2H3 cells, and its absence is a likely explanation for the lack of response of the cells to LPS stimulation.

The releasing media composition was found to be crucial for optimal degranulation. We could show significant differences between EBSS and BSS which are most likely due to a difference in $[Ca^{2+}]$ in the two media. Moreover, the presence of BSA was crucial for a successful experiment, particularly, when BSS was used. The responsiveness of RBL-2H3 cells to immunological stimuli seems to be highly dependant on experimental conditions; it appears clear how different protocols and culture conditions may lead to different results, partially explaining the inconsistencies found in the literature.

Despite the fact that RBL-2H3 cells have commonly been reported to be a mucosal mast cell line, it has to be remembered that they are derived from a histamine releasing subline of RBL-1, a cell line generated from a basophilic tumour and presenting typical characteristic of basophilic cells [28, 29]. We have shown that the expression pattern of TLR4 and CD14 resembles that reported for basophils and it is interesting to speculate that RBL-2H3 share some characteristics of basophils rather than those of mast cells. While this cell line may be a good model for the study of secretory cells, it appears important to highlight that data from degranulation studies using RBL-2H3 cell line should be interpreted with necessary care.

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