

Murine and human mast cell express acetylcholinesterase

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Abstract Expression of catalytically active protein was detected in a murine mast cell line. The primary type of AChE mRNA produced by these cells was found to be the brain and muscle type by PCR amplification of alternative exons from the 3' of mast cells AChE cDNA. AChE was further found to be expressed in the HMC-1 the human mast cell precursor line. Furthermore, utilizing the single cell RT-PCR method we detected AChE mRNA expression in FcεRI-positive single cells derived from human colonic mucosal biopsies. Our findings predict the involvement of mast cell AChE in neuronal-mast cell interactions.

Key words: AChE; Mast cell; Single-cell RT-PCR; Human precursor cell; Alternative splicing

1. Introduction

Mast cells are positioned in close association to peripheral nerves in many tissues [1]. Using an ultrastructural approach, intimate interactions were observed between cholinergic nerves and mast cells both in the rat colon and in the hyperplastic neuronal network characteristic of Crohn's disease in humans [1,2]. As mast cells are present in such proximity to neurons, mast cell–nerve interactions may have important physiological functions. Recent studies have indeed shown that mast cells affect neuronal growth and polarity states and may induce the formation of contacts with nerves through long neurites [3]. In addition, in immunized guinea pigs, histamine and other mast cell granule factors were found to increase acetylcholine (ACh) secretion by cholinergic intestinal nerves through allergic responses [4]. Reciprocally, ACh has been shown to induce mast cell degranulation and vice versa [5]. The mutual interactions between these two cell types might hence play an important role in the pathogenesis of several diseases such as Crohn's disease and cholinergic urticaria [1,6–8].

Nerve cell terminals reaching neuromuscular junctions and cholinergic neurons of the central nervous system secrete the ACh hydrolysing enzyme acetylcholinesterase (AChE). This enzyme is responsible for terminating ACh effects on the post-synaptic membrane [9] and can be linked to the cell membrane by different modes of attachment [10,11]. Within cells of the hematopoietic system, AChE is synthesized and secreted either in a hydrophobic phosphoinositide (PI)-linked form, associated with the external cell surface or in globular hydrophilic forms [9–13]. These forms of AChE were shown by molecular

cloning to differ from each other at their 3'-regions and encode distinct C-terminal peptides [12,13]. The mature subunit of the hydrophilic enzyme is 583 amino acids (aa) long and essentially identical to the brain enzyme [13]. The amphiphilic PI-linked form expressed in erythrocytes deviates from the other starting at aa 544 and is 24 aa smaller in size [13]. A third secretory 'readthrough' form of AChE, identified by molecular biology means, is predicted to be yet smaller in size, as its 26 aa long C-terminus, which deviates from the two others at the same point, lacks the capacity of (PI)-linkage [14]. All three forms hydrolyze ACh rapidly, as was shown by enzyme purification [11] and cDNA transfection studies [14]. In the present study we demonstrate the expression of AChE in both human and mouse mast cells. The predominant mRNA type produced in mast cells is that one coding for the brain and muscle hydrophilic form of this enzyme.

2. Materials and methods

2.1. Cell culture

IL-3 dependent mouse fetal liver-derived mast cells (MC-9) [15] were maintained in RPMI-1640 supplemented with 2 mM L-glutamine, 2 mM non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Grand Island, NY), 50 µM β-mercaptoethanol (Fisher Scientific, Medford, MA), 20 U/ml synthetic IL-3 and IL-4 (kindly provided by I. Clark-Lewis, Vancouver, Canada) with 10% fetal calf serum (FCS) (Bio-Lab, Jerusalem, Israel) or Gro-1 (FCS medium replacement, Biological Industries, Beth Haemek, Israel). Human immature HMC-1 mast cells kindly provided by Joseph H. Butterfield, Mayo Foundation (Rochester, MN) were maintained in RPMI-1640 similarly supplemented to the MC-9 medium but without IL-3 and IL-4.

2.2. Isolation and enrichment of human colonic mast cells (approved by the local human subject committee, the 'Helsinki' committee)

Normal mucosal biopsies incubated in saline overnight at 4°C were cleaned from mucus, incubated in RPMI-1640 medium supplemented with 25 mM HEPES and 1 mM dithiothreitol [16] and treated with 30 U collagenase (Sigma, Israel). Percentage of mast cells out of suspended cells filtered through a nylon wool column was determined by toluidine blue staining. Single cells were transferred in 10 µl of RPMI-1640 25 mM HEPES into individual wells of Terasaki microtiter plate and were incubated for 6 h in 5% CO₂ at 37°C [17].

2.3. RNA isolation and manipulation and RT-PCR

RNA was isolated from 5 × 10⁶ cells by RNA extraction-lysis buffer and centrifugation with CsCl [18]. Reverse transcription (37°C for 60 min) was done with with 200 U of Moloney murine leukemia virus reverse transcriptase (RT) (Clontech, Palo Alto, CA, USA) and 5 µM oligo(dT) primer, reaction buffer, 0.5 mM dNTP and 5 U of RNase inhibitor in 20 µl final volume. For PCR analysis, 2.5 U of Taq DNA polymerase, 3 µM of the appropriate PCR primers, 0.25 mM dNTP, and reaction buffer (Perkin-Elmer, Norwalk, CT) were added to 100 ng of total cDNA from each sample in a programmable thermal controller (MJ Research Inc.) all as described [17].

Single cells were lysed in 100 µl 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% SDS and 100 µg proteinase K (10 min, 37°C), after which 18 µl 3 M NaCl and 50 µg oligo(dT) cellulose were added. Following overnight incubation at room temperature, the

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Abbreviations: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; ACh, acetylcholine; ATCh, acetylthiocholine.

oligo(dT) cellulose was washed thrice with 0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.1% SDS and once with 0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.1% SDS. RNA was eluted with 50 μ l of 10 mM Tris-HCl pH 8.3 (10 min, room temp.), ethanol-precipitated in the presence of 5 μ g tRNA, spun and washed with 70% ethanol. For PCR analyses dried RNA pellets were redissolved in RT buffer containing RNase inhibitor.

2.4. Southern blot hybridization

Amplified cDNA electrophoresed on agarose gels was transferred to Hybond-N membrane (Amersham, Arlington Heights, IL), hybridized with a 32 P-labelled probe overnight, washed at 65°C for 20 min in $0.1 \times$ SSC containing 0.1% SDS and autoradiographed.

2.5. PCR primers

The following PCR primers were employed: α Fc ϵ RI primers: 106+ 5'-ATG GCT CCT GCC ATG GAA TCC CCT ACT-3'; and 519- 5'-GGT TCC ACT GTC TTC AAC TGT GGC AAT-3' [17]. Human AChE (exon 2+) 5'-GCT CGG CCG CCT CAG ACG CCG and (exon 3-) 3'-TCC TCG CTC AGC TCA CGC TTG GG [13]. Murine AChE primers (exon 4) 1671+, 5'-CCA GAC CTG CGC CTT CTG GA; (exon 6) 1841-, 3'-GGT CTG AGC AGCGCT CCT GC; (exon 5) 108- 3'-GAA GCC CGG AGT GGA GGA GG. Orientation of the primers is noted by + or - signs.

2.6. 32 P probes

One picomole of a 25-mer oligodeoxynucleotide (murine AChE, exon 4) 1691–1713 5'-ATC GCT TTC TCC CCA AAT TGC TCA G was 3' end labeled with [32 P]dCTP by terminal deoxy-transferase (USB, Cleveland, OH, USA) [18]. cDNA probes for human and mouse α Fc ϵ RI, kindly provided by Dr. J.P. Kinet (National Institutes of Health, Bethesda, MD), were random prime labelling technique [18] up to a specific activity of 3×10^8 cpm/ μ g. AChE cDNA from human peripheral blood, was PCR amplified as above and 32 P-labeled by random priming. Probes were hybridized at final concentrations of 5×10^6 cpm/ml.

2.7. Immunoprecipitation

Five million cells washed once with 1 ml of DMEM-methionine-free medium (Biological Industries, Beth Haemek, Israel), were incubated (4 h, 37°C) in 5 ml medium containing 500 μ Ci [35 S]methionine (Amersham). For immunoprecipitation cells were lysed in 500 μ l cold lysis buffer (0.01 M Tris-HCl pH 7.4, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 0.15 M NaCl and 0.25 mM PMSF), homogenized, microfuged (30 min at 4°C) and supernatants collected at 4°C. Immunoprecipitation (overnight, 4°C) AE-2, a mouse monoclonal antibody against human AChE (kindly provided by Dr. B.P. Doctor, Washington, DC) or rabbit polyclonal antibody against human AChE (kindly provided by Dr. A. Shafferman, Israel Institute for Biological Research, Ness Ziona, Israel [19]), either preincubated or not with human AChE. Protein-A Sepharose beads (10 mg/tube) (Sigma) were then added (3 h with agitation, 4°C). Resultant immunocomplexes were boiled (10 min in 0.5% SDS) and diluted five-fold with SDS-free buffer. Equal 35 S counts from each mixture were reprecipitated using the same antibody. Precipitates boiled in Laemmli sample buffer containing 0.5% SDS, were electrophoresed on discontinuous 10% acrylamide-bisacrylamide SDS slab gels and exposed to Kodak X-Omat AR film at -70°C for noted times.

2.8. Measurement of AChE catalytic activity

For catalytic activity measurements, cells were grown for 14 days with FCS-free medium, washed with phosphate-buffered saline and homogenized in 200 μ l for 10^6 cells of 0.01 M Tris-HCl, 1 M NaCl, 1% Triton X-100, 1 mM EGTA pH 7.4. Homogenates were microfuged (20 min, 4°C), and clear supernatants were collected. Spectrophotometric measurements of acetylthiocholine (ATCh) or butyrylthiocholine (BTCh) hydrolysis were assayed as previously detailed [13].

3. Results

3.1. Cholinesterase activity

Both acetylthiocholine (ATCh) and butyrylthiocholine (BTCh) were hydrolysed by mast cell extracts at rates of 1.2 and

1.3 μ mol/min/ 10^6 cells, respectively. The ATCh hydrolysing activity was partially sensitive to the selective AChE inhibitor BW284C51 (40% at 1 μ M) and resistant for the BuChE-selective inhibitor iso-OMPA (10% inhibition at 10 μ M). This identified ca. 40% of this activity as AChE. The BTCh activity was sensitive to iso-OMPA and insensitive to BW284C51, as expected from mammalian BuChE (Table 1). Therefore mast cells were shown by this analysis to express both AChE and BuChE and in addition, yet unidentified protein(s) capable of hydrolysing ATCh.

3.2. Protein expression

To clearly demonstrate that mast cells indeed produce AChE and to exclude the possibility that this enzyme originated in serum, we used 35 S labelling followed by immunoprecipitation. MC-9 cells produced polypeptides which were immunoprecipitable with either rabbit polyclonal antibodies or mouse monoclonal antibodies against human AChE (Fig. 1). Moreover, preincubating the polyclonal anti-AChE antibodies with unlabeled human AChE, prior to the addition of the antibody to the lysate prevented the immunoprecipitation, confirming the identity of the precipitated protein as AChE [19]. Gel electrophoresis and autoradiography revealed two main bands, one at a size between 55 kDa and 60 kDa and the other at a size over 120 kDa. Interesting, several other weaker bands were observed at around 70 kDa.

3.3. Type of MC-9 AChE

Alternative splicing yields several mRNA products of the AChE gene [10–14]. This includes three splicing patterns at the 3' region of the gene, leading to the expression of 3 protein isoforms that differ in their C-terminus. The AChE mRNAs produced by murine mast cells were identified by RT-PCR analyses using exon-specific oligonucleotide primers from the consensus regions of exon 4, 5 and 6 (Fig. 2). The cDNA-amplified products were then subjected to DNA blot hybridization analysis using a 25-base oligomer probe from the end of the common region of exon 4. Primers 4 and 6 yielded with MC-9 mRNA a single 170 bp band (Fig. 3A). This band fits the expected size of the amplified product of exon 4–6 splicing pattern. Digestion with *Sac*I led to the disappearance of the 170 bp band and the appearance of a shorter band, hence a more diffuse one, at a size of around 90 bp (Fig. 3B). As shown in Fig. 2, when using primers 4 and 6 for the PCR reaction, the splicing pattern of exon 4–5 should cause the appearance of a band at a size of 833 bp. However, such an amplified product from MC-9 mRNA was not detected. Our inability to detect the amplified product of 833 bp could be due to inefficient amplification of this longer fragment compared with that of the shorter 170 bp fragment when using the same oligonucleotide primers. Therefore, an additional primer from exon 5 was used with

Table 1
Cholinesterase activities of mast cell extracts

Substrate	ATCh	BTCh
mOD/min	0.947	0.340
Hydrolysis rate (μ mol/min/ 10^6 cells)	1.23	1.30
BW284C51 [1 μ M] (% inhibition)	40	none
iso-OMPA [10 μ M]	10	98

*Average of duplicate measurements at several time points.

primer 4 in search for the 170 bp amplification product representing exon 4–5 splicing (Fig. 2). Using these primers the expected 170 bp amplification product was detected using mRNA derived from murine B cells, confirming the efficacy of this primer pair. However, this band was not detected with the same primers when mRNA from MC-9 cells was used (Fig. 3C). Thus, it can be concluded that AChE mRNA in MC-9 murine mast cells is primarily spliced with the Exon 4–Exon 6 pattern and does not contain exon 5.

To determine whether AChE is expressed in human mast cells, mRNA was extracted from the human mast cell precursor cell line (HMC-1), reverse transcribed and amplified with human AChE specific primers. The amplified cDNA products were then subjected to DNA blot hybridization using a human AChE cDNA probe. As can be seen in Fig. 4, a band of the size of approximately 400 bp was detected as expected for the AChE cDNA amplification product.

Thus human precursor mast cells also express the brain and muscle form of human AChE mRNA.

3.4. Expression of AChE in human intestinal FcεRI positive cells

In search of AChE mRNA in non-tumor, mature human mast cells *in vivo*, we examined whether AChE is expressed in

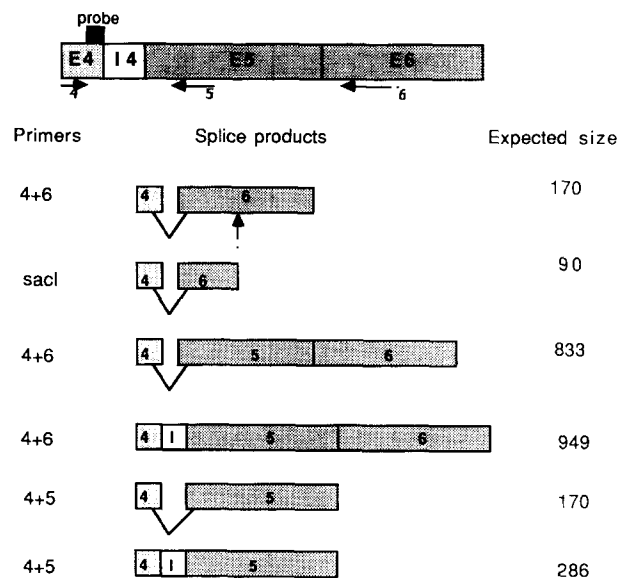


Fig. 2. Exon–intron organization and putative PCR products of the 3' part of the murine AChE gene. Potential splice patterns of the 3' region of the gene are presented. Arrows present the location of the different PCR oligonucleotide primers. The probe is a 25-mer oligonucleotide. The size of the different possible splicing products are noted.

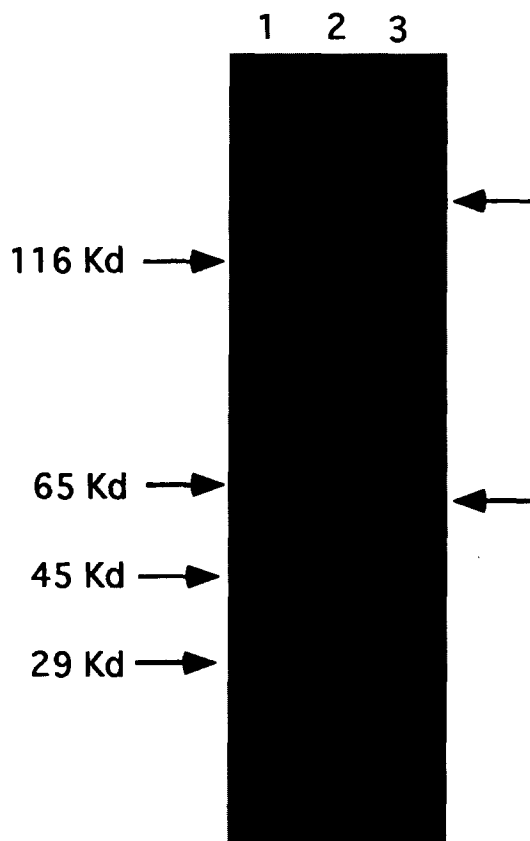


Fig. 1. Identification of AChE in MC-9 cells. [35 S]Methionine labeled MC-9 cells were lysed with lysis buffer as described in section 2. Immunoprecipitates of MC-9 cell extracts were prepared with either polyclonal rabbit anti-AChE (lane 1) or mouse monoclonal anti human AChE antibody (lane 3). Lane 2 = immunoprecipitates obtained with rabbit anti-AChE preincubated with unlabeled human AChE prior to the addition of the antibody to the cell lysates. One representative experiment out of three.

FcεRI-positive cells derived from human colonic mucosal biopsies. Mast cells and other FcεRI positive cells which are involved in the allergic reaction in the intestine are dispersed between other cells in the tissue. Therefore, an enrichment of mast cells was performed. Using a published procedure for isolation of intestinal mast cells with nylon wool [16], positive toluidine blue staining was detected in only 2% of the isolated cells (data unshown). Therefore, this procedure was modified by the addition of overnight preincubation of the tissue at 4°C prior to the enzymatic digestion. Using this modification, 20% of the nylon wool column eluted cells were positively stained with toluidine blue. The RT-PCR single cell technique was thereafter used in order to determine the expression of AChE by individual cells [17]. Each single cell was then transferred into individual wells of Terasaki microtiter plate. The presence of a single cell in each well was verified by carefully scanning the wells with an inverted microscope. Messenger RNA was extracted from each cell and reverse transcribed with primers for the human FcεRI α and the AChE genes. The cDNAs were then amplified by PCR. Bands at the sizes predicted for the amplified cDNA were obtained at around 500 bp and 400 bp for αFcεRI, and for AChE, respectively (Fig. 5). The presence of AChE mRNA was observed in 7 out of 10 αFcεRI positive cells obtained from 3 different human colonic biopsies. We therefore concluded that AChE is most probably expressed in human intestinal mast cells. As the primers employed were from exons 4 and 6, this analysis further demonstrated a single primary splicing pattern for AChE mRNA from precursor and mature mast cells, i.e. the splicing pattern leading to the production of synaptic AChE.

4. Discussion

In our present work we provided, for the first time, evidence for the expression of AChE by mast cells. We have initially

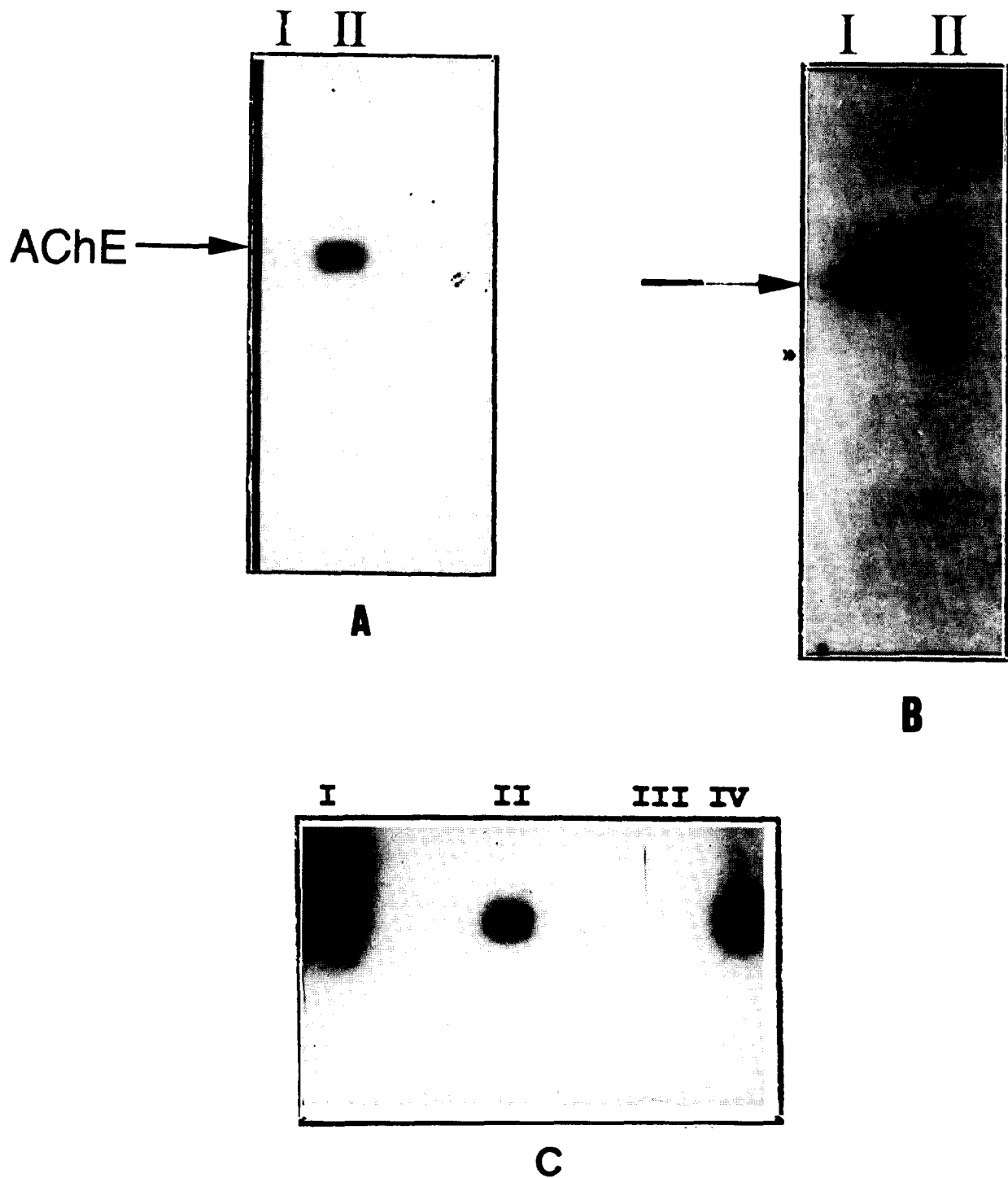


Fig. 3. Exon-intron organization of AChE mRNA in murine MC-9 cells. mRNA isolated from MC-9 cells or murine B cells was reverse transcribed and amplified with oligonucleotide primers for the murine AChE gene. PCR products were separated by agarose gel electrophoresis, transferred to nylon hybridization membrane, hybridized with ^{32}P end-labeled oligo and autoradiographed. (A) MC-9 mRNA amplified with primers 4 and 6. The size of the band is 170 bp. Lane I represents 1/10 the amount of cDNA as compared to II. (B) Digestion of the AChE 4–6 splicing product by *SacI*. The amplification product was digested overnight with 100 units of *SacI* restriction enzyme. Lane I = product of the PCR reaction with primers 4–6; lane II = the same PCR product digested with *SacI*. (C) Amplification with primers 4 and 5. cDNA from murine B cells (I and II) and MC-9 cells (III and IV) was amplified with primers 4–5 (II and III) or 4–6 (I and IV).

detected cholinesterase activity in murine mast cells grown in a serum-free medium. By the use of specific inhibitors we demonstrated that some of the cholinesterase activity was probably due to BuChE activity, and some of it due to AChE activity. The

coexpression of the two cholinesterases in one cell type is not surprising, since in other cells of the hematopoietic system there is also coexpression of both enzyme types [20]. The detected AChE activity level was similar to that reported for leukocytes

[21]. To verify the findings on AChE, which is the enzyme with expected neuronal functions, we searched for its protein and examined splicing pattern of the corresponding mRNA in mast cells.

Immunoprecipitation experiments of nascently labelled protein from MC-9 cells using polyclonal and monoclonal antibodies, revealed a prominent band of between 55 kDa and 60 kDa, several weaker bands above it up to around 75 kDa and a weak band at around 140–150 kDa. The larger band corresponds to the size expected from the dimer forms of AChE. This raised the possibility that although the E4–E5 splicing pattern could not be observed in our RT-PCR analysis, it may exist in mast cells. Alternatively, or in addition, the heavy bands may reflect G₂ dimers of the synaptic enzyme form, with or without structural component(s). The appearance of the most prominent band at around 55–60 kDa and only weaker bands at the expected 70–75 kDa could be due to partial proteolysis of the enzyme, at the C-terminus that occurred during extraction from the protease rich mast cells. Similar proteolysis of AChE at this site was previously observed after digestion with proteinase K [22]. Alternatively, or in addition, it could represent the slightly smaller ‘readthrough’ protein [14]. Selective antibodies should be elicited to resolve this issue.

Several transcripts can be produced by alternative splicing from the AChE gene [9,10,12–14]. These include three alternative splicing patterns at the 3′ region of the mRNA, leading to the production of three potential proteins differing in their C-termini [9,14]. One of these mRNAs encodes a phosphoinositide-membrane anchored protein, the main isoform expressed in erythrocytes. The second codes for the brain and muscle protein, which is probably secreted and can form variable multimeric subunits, whereas, the third protein can only be secreted as monomers [14]. We found that the main transcript in mouse mast cells, like those of the hemopoietic cell lines [14], is the brain one, which fits the splicing pattern connecting exon 4 to 6 and skipping exon 5. The protein coded by this mRNA can be secreted from the cells or be externally associated with the

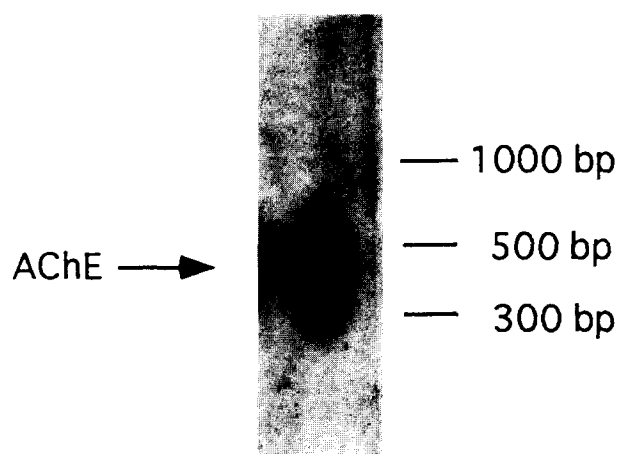


Fig. 4. Expression of AChE mRNAs in HMC-1 human immature mast cells. mRNA isolated from around a hundred HMC-1 cells (using the method employed to isolate mRNA from single cells) reverse transcribed with human AChE primers, and then amplified. PCR products were separated by agarose gel electrophoresis, transferred to nylon hybridization membrane, hybridized with ³²P-labeled cDNAs for human AChE and then autoradiographed for 17 h.

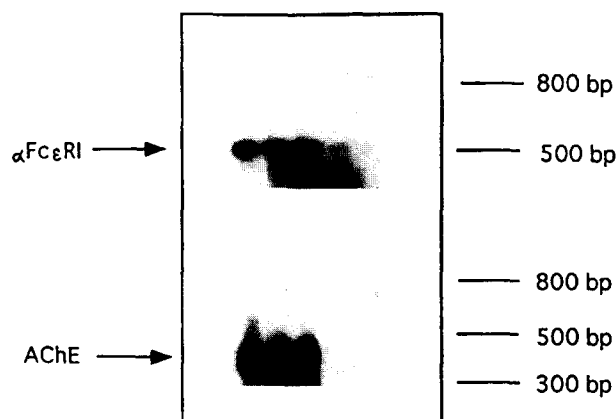


Fig. 5. Expression of AChE mRNAs in FcεRI positive cells from human colonic mucosa. Single cells from mast cell-enriched fractions of human colonic mucosal biopsies were carefully divided into Terasaki plates. mRNA from each of the single cells was individually reverse-transcribed with human αFcεRI and AChE primers and then PCR-amplified (30 cycles). PCR products were separated in agarose gels by electrophoresis, transferred to nylon hybridization membrane, hybridized with ³²P-labeled cDNAs for human αFcεRI, and AChE and autoradiographed for 17 h.

cellular membranes. However, the main polypeptide immunoprecipitated from mast cells was somewhat smaller from the brain and muscle form peptide, either through proteolytic cleavage or, perhaps, reflecting the readthrough protein. Moreover, our immunoprecipitation data demonstrated the presence of dimers, which raises the possibility that very low levels of mRNA with the exon 4 to exon 5 splicing pattern also exist in these cells, producing GPI-linked dimers. This analysis therefore, leaves the question of the exact nature of the mast cell AChE open.

Next we decided to examine whether AChE is expressed in human mast cells. First, AChE mRNA was detected in a human mast cell precursor line (HMC-1). In view of the considerable heterogeneity between mast cells from different organs [23], and since mast cell neuronal interactions were observed in the intestine [2,24], we searched for AChE expression in human intestinal-derived FcεRI cells by using the single cell RT-PCR methodology. We observed a close correlation between the percentage of the toluidine-positive cells and the percentage of cells expressing FcεRI, which sustains the assumption that the majority of the isolated FcεRI-positive cells were mast cells. Furthermore, we find that cells isolated by the same method contain the *c-kit* mRNA which is known to be expressed in mast cells but not in other FcεRI expressing cells such as basophils (Ligumski, M., Kuperstein, V., Nechushtan, H. and Razin, E., unpublished results). AChE mRNA was expressed in most (7 out of 10) of the FcεRI-positive cells derived from three different human colon biopsies. Therefore AChE is most probably expressed in human intestinal mast cells.

Currently we can only speculate on the physiological role played by AChE expressed by mast cells. In other non-neuronal cells synthesizing AChE, such as myocytes, this enzyme is presumably responsible for the degrading of ACh released by cholinergic neurons [9,10]. A similar role for mast cell AChE seems logical. Several works demonstrated that ACh induced mast cell degranulation can take place only in immunologically stimulated cells (for a detailed discussion of this issue see [25]).

In addition, it was shown that antigenic stimulation that led to the degranulation of mast cells caused an increase in ACh release from intestinal nerve cells [4]. Thus a possible positive feedback cycle can exist: activation of mast cells by antigen can increase ACh release by neurons, and this response may lead to further degranulation of mast cells.

Interactions between mast cells and neurons can possibly contribute to the pathogenesis of many diseases, including allergic and chronic inflammatory ones like Crohn's disease [1]. The most direct link between a disease and mast cell–neuron interactions has been shown for cholinergic urticaria. In one patient with this disease, the immediate type hypersensitivity reaction to copper was found to be correlated with increased mast cell ACh receptor concentrations [8].

Acetylcholine levels in the extracellular space are controlled not only by the amount released from nerve cells, but also by the rate of its degradation, mainly by AChE. Therefore, mast cell-produced AChE can serve an important role in the modulation of mast cell–neuron interactions. Contribution to our understanding of the pathogenesis of diseases linked to neuro–allergic interactions might be achieved through the study of mast cell produced AChE from individuals with these diseases.

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