

Mast cell degranulating peptide binds to RBL-2H3 mast cell receptors and inhibits IgE binding

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Abstract

Fluorescent and biotinylated analogs of mast cell degranulating (MCD) peptide were synthesized and the labels fluorescein isothiocyanate and N-hydroxysuccinimidobiotin were conjugated at position 1 in the MCD peptide sequence. The analogs with these moieties retained histamine-releasing activity as high as that of the parent MCD peptide in rat peritoneal mast cell assays. These labeled analogs were used in rat basophilic leukemia cells (RBL-2H3) to demonstrate by confocal microscopy and flow cytometry the specific binding of MCD peptide to mast cell receptors. Consequently MCD peptide was found to compete with and inhibit the binding of fluorescent IgE on RBL cells as monitored by flow cytometry. Thus MCD peptide may prove to be useful in the study of IgE receptor-bearing cells. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

Mast cells are the main target cells for immunoglobulin E (IgE) molecules which bind with high affinity to FcεRI receptors on the mast cell surface. When receptor-bound IgE molecules are cross-linked by multivalent allergens, the receptors are activated and cause the mast cell to degranulate and release histamine and other inflammatory mediators [25,33]. Mast cell degranulation can also be directly non-IgE-mediated [18,34]. In any case, the release of these mediators initiates the symptoms of type I hypersensitivity allergic reactions [1]. Over the years, peptides from IgE or its FcεRI receptor have been designed and studied as inhibitors of the IgE/FcεRI interaction [15,16,31]. These efforts showed that basic peptides and cyclic disulfide bonded peptides had higher binding specificity and affinity for the IgE receptor than the corresponding linear ones [22]. Mast cell degranulating (MCD) peptide is a natural compound found in bee venom with such properties. It is one of the strongest natural histamine secretagogues, stimulating mast

cell degranulation at low concentrations [20]. At higher concentrations it has been found to inhibit mast cell degranulation [4,21]. It may be hypothesized that these two antagonistic activities are attributable to (1) MCD peptide's direct non-IgE-mediated interaction with the mast cell surface and (2) to its IgE-mediated actions. The latter involves cross-linking with IgE molecules or interaction with IgE via disulfide exchange complexes [5]. Thus MCD appears to be a suitable compound for studying allergic processes. The postulation of a natural mast cell receptor is needed for establishing MCD peptide's mechanism of action. The structural features and biological properties of MCD peptide suggest that mast cell activation by this peptide may be consistent with a receptor mediated mechanism [23].

The availability of the rat basophilic leukemia mast cell line RBL-2H3 allowed us to initiate receptor binding studies. These cells can be cultured in large numbers and generally have a lower histamine-releasing activity than normal mast cells [3,40]. RBL-2H3 cells are homologous with rat mucosal mast cells and bind IgE with high affinity [28,29,40]. They are therefore used as a standard model for mast cell secretory and IgE receptor characteristics.

To identify peptide receptors, labeled ligands are needed. Commonly used radiolabeled peptides are, in the case of

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MCD peptide, not readily feasible [42]. A better alternative for characterization and cellular localization of MCD peptide receptors is the use of fluorescent and biotinylated derivatives. Based on our structure-activity studies with MCD peptide [10–12], we chose position 1 in the MCD peptide sequence to attach the fluorescence and biotin labels. The resulting fluorescent (FITC) and biotinylated (bio) MCD peptide analogs retained full biological activity. In the present communication, we present data to show that these labeled MCD peptide analogs allowed localization and binding to the mast cell receptor on RBL-2H3 cells as seen by confocal microscopy and flow cytometry. In this context unlabeled MCD peptide was also tested for its ability to compete with IgE for receptor binding sites on the RBL-2H3 cells.

2. Methods

2.1. Synthesis of fluorescent and biotinylated MCD peptides

The FITC- and bio-MCD peptides were prepared by stepwise solid phase synthesis on an MBHA (p-methylbenzhydrylamine) resin using Boc/benzyl protected amino acids (Novabiochem). The otherwise fully protected peptides on the solid support were acylated in the only deprotected N-terminal amino group with fluorescein 5-isothiocyanate (FITC) or N-hydroxysuccinimidobiotin (Pierce) in dimethylformamide and the pH was adjusted to 8.5 with N,N-diisopropylethylamine. The completion of the reaction was evaluated by the Kaiser test [26]. Deprotection, oxidation, purification, and analysis of the peptides followed a well established protocol for MCD peptide analogs detailed previously [7,10–12].

2.2. Cell culture

The basophilic leukemia RBL-2H3 cell line [3] was a generous gift from Dr. D. Holowka, Cornell University. The cells were grown on tissue culture plates in minimal essential medium (MEM) with Earle's salts supplemented with 1% insulin-transferrin-selenium A, 2% L-glutamine, 0.1% gentamicin (all from Gibco), and 20% fetal bovine serum (Hyclone) in an incubator with a humidified atmosphere (95% air, 5% CO₂) at 37°C. RBL-2H3 cells were readily detached from these plates by gentle scraping and allowed to rest for 20 min in an incubator at 37°C. They were then centrifuged at $200 \times g$ for 5 min and washed twice with phosphate buffered saline (PBS, pH 7.8) containing 0.2% bovine serum albumin (BSA). After the final centrifugation, the cells were suspended in the same medium to a final concentration of 2×10^6 cells/ml.

2.3. Flow cytometry

Flow cytometry was performed using an FACScan flow cytometer (Calibur, Becton-Dickinson). Cell aliquots (6 to 7×10^4 cells) were incubated at room temperature in 50 μ l final volume. At the end of the incubation, cells were pelleted, washed, resuspended in PBS/BSA to 500 μ l and analyzed with the flow cytometer. Cell suspensions without labeled peptides were used as controls. All experiments were repeated at least four times. Three kinds of experiments were performed:

1. For saturation experiments, cell suspensions were incubated at increasing concentrations of FITC-MCD peptide (20 nM to 1 μ M) for 30 min. In the case of bio-MCD peptide, after 30 min initial incubation 2.5 μ l FITC-avidin from a 1 mg/ml dilution (Molecular Probes) was added and the incubation continued for another 30 min.
2. For binding experiments, cells were incubated for 30 min with 10^{-6} M FITC-MCD peptide or bio-MCD peptide together with 2.5 μ l FITC-avidin in the presence or absence of 10^{-4} M unlabeled MCD peptide.
3. For competitive binding experiments, FITC-IgE [17] or Alexa Fluor-IgE was used (provided by Dr. D. Holowka and Accurate Chemicals). The latter dye is modified only in the FITC moiety and has the same absorption/emission maxima as FITC (Molecular Probes). It is, however, more photostable and less pH sensitive than FITC. Cells were preincubated with various concentrations of MCD peptide (1 nM to 1 mM) for 30 min followed by the addition of 20 nM fluorescent IgE and an additional incubation of 30 min.

2.4. Fluorescence microscopy

2×10^5 cells/ml were grown in supplemented MEM to 50% confluence on polylysine-coated glass coverslips. The cultured coverslips were washed with plain MEM and incubated in the dark in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (with L-glutamine and 15 mM Hepes without NaHCO₃, Sigma) with 10^{-6} M FITC-MCD peptide in the presence or absence of 10^{-4} M unlabeled MCD peptide. The incubation times were 20 or 45 min at 0°C and 30 or 45 min at 37°C. The cells were then fixed with methanol at –20°C for 10 min, rinsed with PBS and mounted for viewing in glycerol based medium with a confocal laser scanning microscope (Leica).

3. Results

3.1. Preparation of labeled MCD peptide analogs

The fluorescence and biotin markers were covalently bound to the N-terminus of the MCD peptide while it was

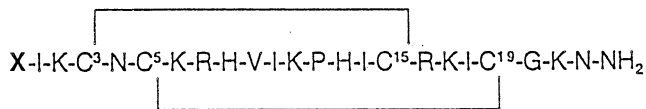


Fig. 1. Amino acid sequence of MCD peptide. Lines connecting cysteines represent the disulfide bonds. X marks the site of the fluorescence (FITC) or biotin (bio) labels.

still attached to the solid support. The remaining positively charged amino groups in the sequence (net positive charge of MCD peptide +10) were protected. This strategy facilitated the first synthesis and analysis of these two MCD peptide ligands. The established synthetic protocol for MCD peptide including hydrogen fluoride cleavage, during which the prosthetic groups remain stable, could be retained [7,10–12]. Consequently, the tedious resolution of multiple monoacylated or polyacylated labeled derivatives was avoided. The monoderivatized isoleucine¹-FITC and -bio analogs of MCD peptide (Fig. 1) were also tested for the first time for biological activity. They were fully active in the histamine-release assay compared to the activity of unlabeled MCD peptide by using the same protocol as with other previously synthesized unlabeled MCD peptide analogs [13]. The ED₅₀ values for histamine-releasing activity of the peptides were as follows: MCD, 1.12×10^{-5} M; FITC-MCD, 0.84×10^{-5} M; bio-MCD, 0.66×10^{-5} M.

3.2. Binding of fluorescent and biotinylated MCD peptides and inhibition of FITC-IgE binding: flow cytometry

Because there is little available information about MCD peptide mast cell receptors, we initiated binding studies with the labeled MCD peptide analogs and RBL-2H3 cells using flow cytometry. Fig. 2 demonstrates a concentration dependent binding of FITC-MCD peptide (A) and bio-MCD peptide (B) to the cells starting at low nanomolar concentrations and extending to complete saturation at 1 μ M peptide.

The ability of the labeled analogs to recognize cell surface receptors was measured as the mean fluorescence intensity of the cells at different concentrations of labeled peptides in the absence (total cell bound fluorescence) or presence (non-specifically bound fluorescence) of unlabeled MCD peptide. A typical fluorescence histogram, shown in Fig. 3, illustrates the binding of FITC- and bio-MCD peptides at 20 nM concentration where the best ratio of total to non-specific fluorescence was obtained. The cells were incubated with 20 nM FITC-MCD peptide (A) or 20 nM bio-MCD peptide with 2.5 μ l FITC-avidin (B). They showed a shift to greater fluorescence intensity compared to cells incubated without FITC-MCD peptide (autofluorescence) or with FITC-avidin alone. The labeled cells failed to show any increase in fluorescence intensity after addition of 2 μ M unlabeled MCD peptide.

In addition, competitive binding experiments of MCD

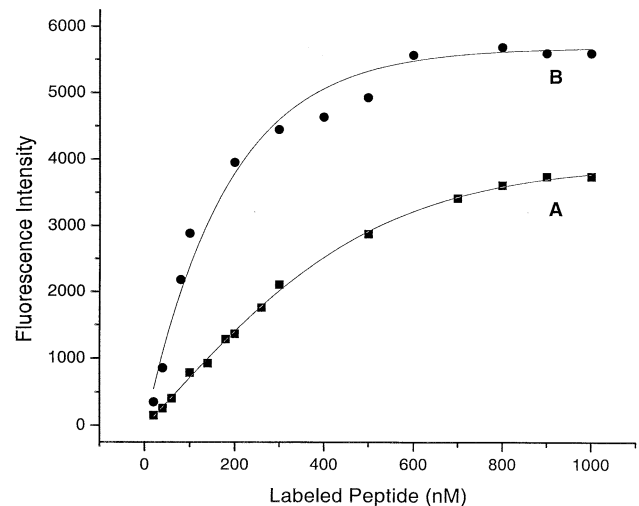


Fig. 2. Saturation of binding of FITC-MCD ligand (A) and bio-MCD ligand (B) to RBL-2H3 cells. Cells were incubated at 22°C with increasing concentrations of A and B for 30 min. Cells incubated with B were additionally incubated with FITC-avidin for 30 min. The fluorescence intensity of the bound ligands was measured by flow cytometry. Data points represent means of four independent experiments.

peptide with IgE were performed. Fig. 4 shows the effects of various dilutions of MCD peptide at a fixed concentration of 20 nM FITC-IgE monitored by flow cytometry. The peptide reduced the binding of FITC-IgE to a maximum of 43% of the control value. The control was defined as the fluorescence intensity of 20 nM FITC-IgE bound to the cells in the absence of peptide (100%) after subtraction of non-specific binding (6%) determined in the presence of an 100-fold excess of rat IgE. The concentration of peptide at 50% inhibition of the FITC-IgE (IC₅₀) was approximated 20 ± 1 μ M. In a similar experiment (data not shown), the modified MCD peptide analog Orn¹⁷-MCD showed about 10% overall lower inhibition. In the same assay the fluo-

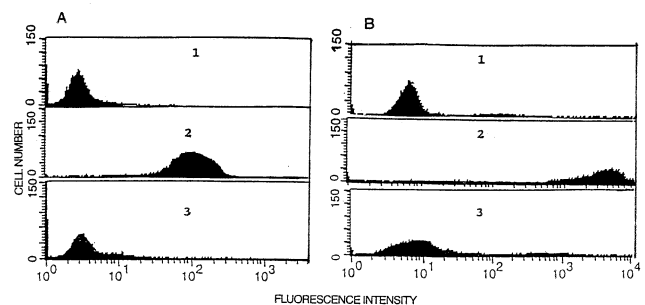


Fig. 3. Flow-cytometric analysis of RBL-2H3 cells using FITC-MCD peptide (A) and bio-MCD peptide with FITC-avidin (B). The number of cells is plotted against the logarithm of fluorescence intensity of the bound ligand. Panel A: trace 1, cells without ligand; trace 2, cells incubated with 20 nM FITC-MCD; trace 3, cells incubated with 20 nM FITC-MCD and 2 μ M MCD peptide. Panel B: trace 1, cells incubated with 2.5 μ l FITC-avidin; trace 2, cells incubated with 20 nM bio-MCD and 2.5 μ l FITC-avidin; trace 3, cells incubated with 20 nM bio-MCD, 2.5 μ l FITC-avidin, and 2 μ M MCD peptide.

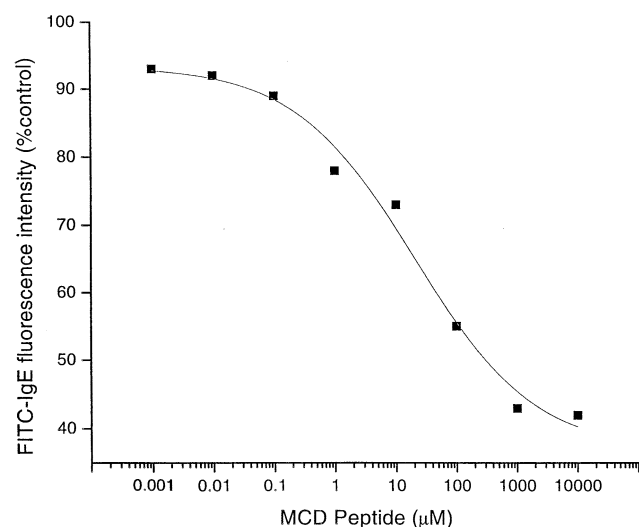


Fig. 4. Inhibition of FITC-IgE binding to RBL-2H3 cells by increasing concentrations of MCD peptide. After preincubation of cells with MCD peptide, fluorescence intensity was measured upon addition of 20 nM FITC-IgE. Results are expressed as the percentage of remaining FITC-IgE fluorescence bound to the cells compared to the control. The control value was the fluorescence intensity of 20 nM FITC-IgE in the absence of peptide corrected for nonspecific binding detected in the presence of 2 μ M rat IgE. Data points represent means of four independent experiments.

rescence intensity of FITC-IgE after addition of 100 nM peptide or 100 nM unlabeled rat IgE (Chemicon) was 266 and 69 fluorescence units respectively. Therefore, on a molar basis the peptide was at least 4 times weaker than IgE in displacing FITC-IgE.

3.3. Binding of fluorescent MCD peptide: fluorescence microscopy

We also used FITC-MCD peptide to examine binding and internalization on MCD peptide in RBL cells by confocal microscopy (Fig. 5). When RBL-2H3 cells were incubated with 10^{-6} M FITC-MCD at 0°C for 45 min in the dark, the fluorescence signal initially was confined to patches on the cell surface and there was no detectable internalization (A). Then the temperature was raised to 37°C to allow endocytosis to proceed. After cells were incubated for 20 min at 0°C and 30 min at 37°C the fluorescence staining was seen in small vesicles beneath the plasma membrane (B). After 45 min at 37°C the vesicles were more centrally located in the cytoplasm (D and E). Binding and internalization could be reversed by addition of a 100-fold excess of unlabeled MCD peptide (C) after incubation for 20 min at 0°C and for 45 min at 37°C.

4. Discussion

In the present study, MCD peptide receptor binding was demonstrated by flow cytometry and visualization by con-

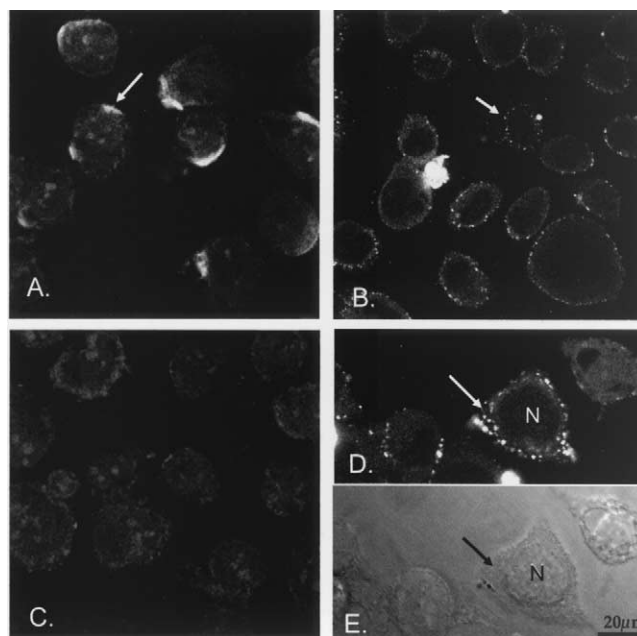


Fig. 5. Confocal microscopic images of the binding and internalization of FITC-MCD peptide in RBL-2H3 cells. Fluorescence of cells seen upon addition of 10 μ M FITC-MCD peptide and (A) incubation at 0°C for 45 min; (B) incubation first at 0°C for 20 min and then at 37°C for 30 min; or (D) incubation at 0°C for 20 min and at 37°C for 45 min. (E) represents the differential interference contrast image of D. Binding and internalization of fluorescent peptide were blocked by a 100-fold excess of unlabeled MCD peptide when cells were incubated as in D. N marks the nucleus.

focal microscopy using fluorescent and biotinylated analogs. Fluorescent and biotinylated analogs were originally used by us to study neurohypophyseal peptide hormones [8,14,27]. This approach has also been successfully applied to a variety of other biologically active peptides [6,19,24,37,43]. The advantages and pitfalls in the use of such labels compared with radioactive labels are well recognized [32]. For example, although receptor binding can be quantified with radioactive ligands, nonradioactive labels allow better cellular localization. On the other hand, the bulky fluorescence and biotinyl moieties may interfere with biological activity due to steric hindrance. Generally, there are inherent problems in labeling MCD peptide. The bicyclic structure of this peptide includes many reactive groups, (i.e., 10 positive charges) and lacks convenient positions for inserting radiolabels [42].

Previous structure-activity studies revealed the importance of the C-terminus of MCD peptide for histamine release. In contrast, the N-terminus, including position 1, could be modified or deleted without loss of activity [7,10–12]. That knowledge and the strategies and efficiency of solid phase synthesis [2] allowed labels to be readily attached only to the N-terminal isoleucine. The resulting mono FITC-MCD and bio-MCD peptides showed histamine-releasing activity similar to the activity of the unlabeled MCD peptide in peritoneal mast cells. In this assay we employed peritoneal mast cells because of their high hista-

mine content and great responsiveness to a wide range of stimuli compared with other mast cell lines [18,40].

The high fluorescence yield of the FITC moiety in basic pH and the high affinity of the bio-group to FITC-avidin allowed the use of FITC-MCD and bio-MCD peptides for binding and internalization studies in RBL cells. The binding of these derivatives followed by flow cytometry was saturable with increasing concentrations of these peptides. This suggests that the binding is receptor mediated.

Flow cytometry also demonstrated binding of FITC-MCD peptide directly and of bio-MCD peptide with FITC-avidin. After addition of these analogs, a large increase in the fluorescence intensity of the cells over the background levels was observed and could be blocked by an excess of unlabeled peptide. These results showed that the labeled analogs bind specifically to the mast cell receptor on RBL-2H3 cells.

Also, we have demonstrated receptor-mediated ligand internalization by endocytosis. Endocytosis is a process important for the functioning and recycling of all membrane receptors [36]. Using FITC-MCD and confocal microscopy, we showed that binding and internalization of the analog was time and temperature dependent, was seen only in internalized membrane limited vesicles in the cytoplasm and could be competed by excess of unlabeled MCD peptide. These properties are characteristic of receptor bound endocytosis.

However, due to the basic nature of MCD peptide one may consider the possibility of endocytosis vs. cell penetration as has been shown for some transcription factor or designed basic peptides of different structural classes [30, 35,38,39]. Some of the physiological effects of these basic peptides are: a) their binding is not saturable with increasing peptide concentrations, b) they translocate across the plasma membrane even at 0°C although endocytosis is inhibited under 18°C, c) their distribution after penetration is detected throughout the cell interior and in the nucleus, and d) they can not be blocked with unlabeled peptides. From these results it is concluded that the cellular uptake of these peptides is not mediated by endocytosis. In contrast our findings with MCD peptide do not fit these criteria. Therefore our results provide direct evidence that MCD peptide binds to RBL cells through a membrane receptor followed by endocytosis.

In view of the binding of MCD peptide ligands to the RBL cells, it appeared relevant to initiate competitive binding studies with labeled IgE, which binds with high affinity ($K_a = 10^{-10} \text{ M}^{-1}$) to its mast cell receptors [41]. Using fluorescent IgE, we have shown that MCD peptide can compete with IgE for receptor binding sites in RBL cells. The specificity of the observed inhibition of IgE binding was supported by the finding that Orn¹⁷-MCD peptide, an MCD peptide superagonist, also had an inhibitory effect. These results are, to a certain degree, comparable with experiments using peritoneal mast cells, radiolabeled IgE, and peptides derived from IgE sequences [15]. In this re-

spect, cyclic peptides from IgE or FcεRI receptor sequences also showed inhibitory activity [16,31]. In this case no comparisons can be drawn because these peptides were tested in cell-free assays. Under our experimental conditions, IgE was 4-fold more potent in inhibiting FITC-IgE binding than MCD peptide. On the other hand, MCD peptide has been found to inhibit IgE-mediated histamine release at high concentration [9]. Taken together these findings suggest that MCD-like peptides may be useful for studying and modulating allergic processes.

Acknowledgments

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