Dansyl cadaverine regulates ligand induced endocytosis of interleukin-8 receptor in human polymorphonuclear neutrophils

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Abstract Interleukin-8 (IL-8), a neutrophil chemotactic agent, acts as a key mediator in a large number of acute and chronic inflammatory diseases. At 37°C, the receptor for IL-8 is rapidly internalized with its ligand. But no specific inhibitor of this ligand induced internalization of the receptor has been reported so far. We have found that monodansyl cadaverine (MDC) inhibited about 70% of IL-8 induced endocytosis and caused 70% and 66% inhibition of IL-8 mediated chemotaxis and respiratory burst response, respectively, in neutrophils. The uninternalized receptor was detected by anti IL-8R antibody in MDC treated cells. The endocytosis of IL-8R was strongly inhibited under Ca^{2+} depleted conditions which was restored on addition of 1 mM $CaCl_2$ indicating the critical involvement of a Ca^{2+} ion in the process. Absence of receptor internalisation makes the MDC treated neutrophils suitable for studying the interaction of IL-8R with potential therapeutic agents e.g. for in vitro screening of anti-inflammatory agents.

Key words: Interleukin-8; IL-8 receptor; Endocytosis of IL-8; IL-8 Internalization; Inflammation

1. Introduction

The cytokine, interleukin-8, is a potent chemoattractant for neutrophils. It belongs to the alpha-subfamily (C-X-C) of chemokine superfamily [1–4] and plays an important role in many immune and inflammatory responses. IL-8 has been reported to be present in high concentration in inflammatory exudates of a large number of neutrophil driven acute and chronic inflammatory diseases and upto 98% of neutrophil accumulation can be reduced by the addition of mAbs of IL-8, suggesting IL-8 to be the principal chemokine responsible for chemoattraction of neutrophils in these diseases [5–7].

IL-8 interacts with the target cell through a specific cell surface receptor which is known to be of two types – type A (59 kDa) and type B (67 kDa). Both the receptors independently mediate chemotaxis, Ca^{2+} mobilization and other related functions of the cells [8,9], and are downregulated at 37°C in presence of its ligand viz. IL-8. The internalized receptors are reexpressed on the cell surface in absence of the ligand. This rapid ligand induced downregulation and subsequent reexpression of the receptor is known to be intimately associated with IL-8 directed migration of neutrophils [10–12]. Receptor-mediated endocytosis is an important phenomenon for a viable eukaryotic cell. The inhibition of the endocytosis process in other cells has been demonstrated by several techniques including biochemical, electron microscopic and fluorescent microscopic techniques [13]. However, until now, no report is available regarding the regulation of IL-8 mediated endocytosis of its receptor on neutrophils. Therefore any information related to the regulation of rapid endocytosis of an inflammatory cytokine like IL-8 seems to be very important.

Monodansyl cadaverine (MDC), a non-toxic agent and a very potent inhibitor of serum and liver transglutaminase, has been widely used for blocking receptor mediated internalization of many peptides, hormones, and growth factors [13,14]. We have found that MDC can inhibit the receptor mediated endocytosis of IL-8 as well as IL-8 mediated biological responses on human neutrophils. The study may be useful for detailed understanding of the endocytic pathway involved in the process and also for exploring the transmission of signals involved in IL-8 mediated biological responses.

2. Materials and methods

Human recombinant IL-8 (2×10^7 U/mg) was a gift from Prof. K. Matsushima, Kanazawa University, Japan and from Dainippon Pharmaceutical Company, Osaka, Japan. Polyclonal human anti-IL-8R antibody was raised in rabbits against 58 kDa receptor protein of human neutrophils, and the anti-IL-8R antibody was characterized by 60–70% binding inhibition of [^{125}I]IL-8 to neutrophils and also by Western blotting analysis [15].

Human neutrophils were separated from fresh peripheral venous blood of healthy donors by dextran sedimentation followed by the Ficoll-Paque centrifugation method [16]. The preparation contained 90–95% PMNs. By Trypan blue dye exclusion method, the viability of prepared neutrophils was examined and about 98% cells were found to be viable.

Human recombinant IL-8 and anti-rabbit IgG antibody were radiolabelled with ^{125}I following the method as described earlier [17]. The specific activity of labelled IL-8 was 3×10^7 to 5×10^7 cpm/μg protein

The biological activity of IL-8 and IL-8 induced migration of neutrophils was examined in a modified Boyden chemotactic chamber (Neuroprobe Inc. Bethesda, MD), using polyvinyl pyrrolidone free polycarbonate filter (10 μm thick, 3 μm pore size) as reported earlier [12].

Respiratory burst response of the neutrophils or oxygen consumption were measured in an oxygraph (Gilson, villiers – Ve-Bel, France) equipped with a Clark electrode. Cells after treatment, were suspended in binding medium (2×10^6 cells in 2.2 ml suspension/tube). Effect of IL-8 was measured following 10 min incubation with 100 ng/ml IL-8 at 37°C with stirring. The respiratory medium for the human neutrophils was RPMI-1640 in 20 mM HEPES (with 0.5% BSA). All readings were taken over a 7.5 min time interval [18].

3. Results

3.1. Effect of dansylcadaverine on internalization of IL-8R

Effects of monodansyl cadaverine on the ligand mediated receptor internalization were examined by first treating human neutrophils with different amounts of MDC for 15 min at 37°C followed by [^{125}I]IL-8 binding at 4°C for 2 h. After washing, the cells were warmed at 37°C for 25 min and [^{125}I]IL-8 bound to

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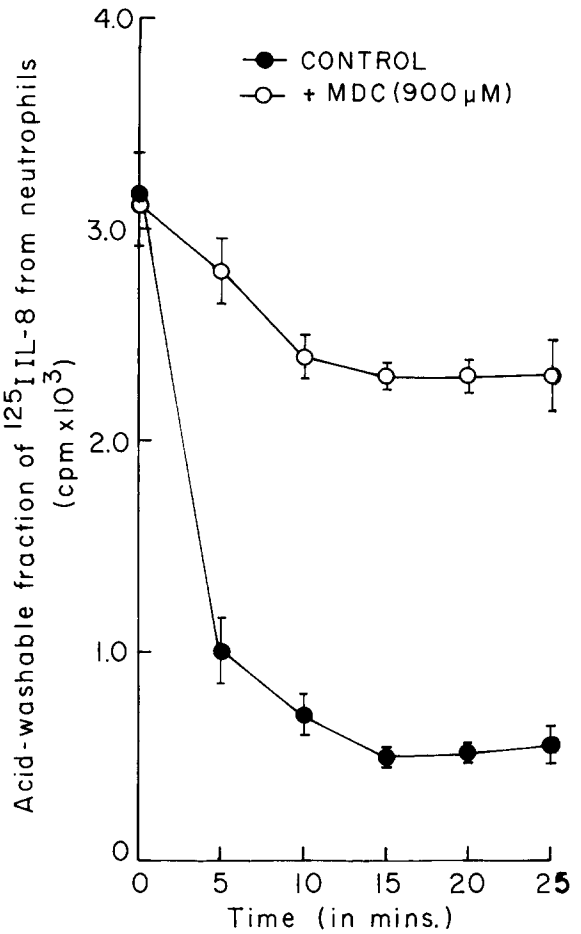

Fig. 1. Effect of dansyl cadaverine on IL-8 induced endocytosis of the receptor in human neutrophils. Freshly separated neutrophils (2×10^6 cells/tube) suspended in 'binding medium' were treated with 900 μM MDC for 15 min at 37°C. Another set without addition of MDC was run parallely. The tubes were cooled, radiolabelled IL-8 was added (4 ng/tube) in each tube and incubated for 2 h at 4°C. Then unbound IL-8 was removed, the cells were warmed at 37°C for various time periods as indicated in the figure and then tubes were treated with acidic glycine solution for 90 s. The acid washable and non-washable fractions were collected and the total [^{125}I]IL-8 content in the tubes were measured by a gamma counter. This figure presents the data of radiolabelled IL-8 present in acid washable fractions of control and MDC treated samples in cpm ± S.D. of duplicate.

cell surface was removed by brief exposure to glycine-HCl solution (pH 3.0, 0.05 M and NaCl, 0.1 M). Measurement of the amount of [^{125}I]IL-8 in supernatant showed about 90% elution of the bound [^{125}I]IL-8 from the control-I cells (incubated at 4°C) and only 15% from the control-II cells (warmed at 37°C for 25 min). [^{125}I]IL-8 eluted from the cell surface considerably increased in MDC treated cells in a dose dependent manner where maximum radiolabelled IL-8 was obtained (70%) with an optimum dose of MDC (900 μM).

The kinetics of internalization of IL-8 receptor was then studied in presence and absence of MDC (900 μM). The elution profile of the control sets, showed a gradual decrease in elution profile of [^{125}I]IL-8 bound to cell surface, with time, whereas in the MDC treated cells, there was, 60–70% more surace elution (Fig. 1).

3.2. Measurement of the IL-8 receptor level

Interleukin-8 receptor level in Control-I, Control-II and MDC treated cells was measured with anti IL-8 receptor antibody followed by binding with [^{125}I]anti-rabbit IgG (raised in goat). For this experiment, Control and MDC treated (500 μM and 900 μM) cells were preincubated for 15 min at 37°C. Then 500 ng/ml unlabelled IL-8 was added in each tube except in Control-I and the incubation was continued for 2 h at 4°C. After washing, the cells were warmed at 37°C for 25 min, then briefly exposed to acidic glycine solution for 90 s, and incubated with anti-IL-8 receptor antibody (1 : 100 dilution) for 45 min at 37°C. The cells were then washed and incubated with [^{125}I]anti rabbit IgG for 1 h at 37°C and the radiolabelled IgG bound to the cells were measured.

The results showed that with respect to Control-I only 28% IgG binding was obtained in IL-8 treated cells whereas 500 μM and 900 μM MDC treated cells showed binding of IgG to be

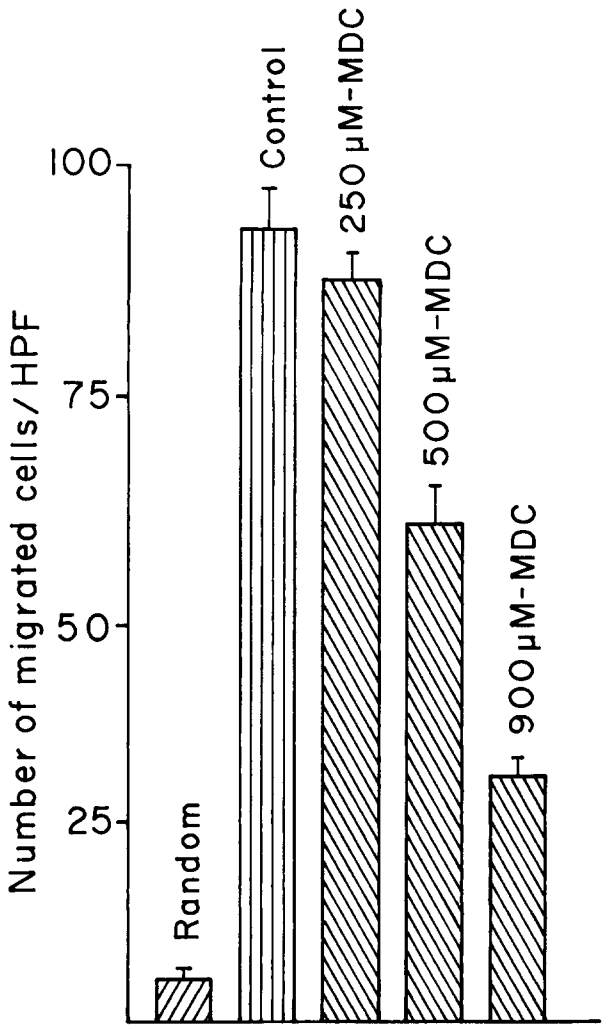

Fig. 2. Effect of MDC on IL-8 induced chemotaxis in human neutrophils. Neutrophils suspended in binding medium were incubated with MDC for 15 min at 37°C. A control set was also run parallelly as before. Then the cells were put in the well (50,000 cells/well) of the upper chemotactic chamber. In the wells of the lower chamber 100 ng/ml IL-8 was added. The migration was initiated by keeping the chamber in a 5% CO_2 incubator (37°C). After 60 min, the membrane was removed, stained with Giemsa and counted under a phase contrast microscope. This figure indicates the number of cells (mean ± S.D.) migrated in control and MDC treated of triplicate samples.

62% and 85%, respectively, upon addition of the same amount of IL-8.

MDC, as such, does not induce F_c receptor expression on the surface of neutrophils as seen in a separate experiment, where MDC treatment was found to have no effect on the binding of [^{125}I]IgG at 37°C to human neutrophils.

3.3. IL-8 induced chemotaxis

To find out whether the IL-8 induced endocytosis of the receptor is essential for directed migration of neutrophils, the cells were incubated with 250, 500 and 900 μM MDC and the chemotaxis examined in a Boyden Chamber. The data presented in Fig. 2 show that MDC inhibits the IL-8 directed migration in a dose dependent manner. The control set showed migration of an average of 93 cells per high power field. Under identical conditions, on an average, 59 and 29 cells migrated in the presence of 500 and 900 μM MDC, respectively (Fig. 2).

3.4. Respiratory burst response

In order to examine the effect of IL-8 mediated endocytosis on the respiratory burst response, we treated neutrophils with 900 μM MDC at 37°C and induced O_2 consumption was measured and compared with that of the control cells (without addition of MDC). Data in Fig. 3 show that in control set, neutrophils consumed 16.36 nM oxygen which was increased to 28.48 nM in presence of 100 ng/ml IL-8 (Fig. 3A,B). On the other hand in the MDC treated cells O_2 consumption was found to be 13.93 nM, which was reduced to 9.67 nM in presence of 100 ng/ml IL-8 (Fig. 3C,D).

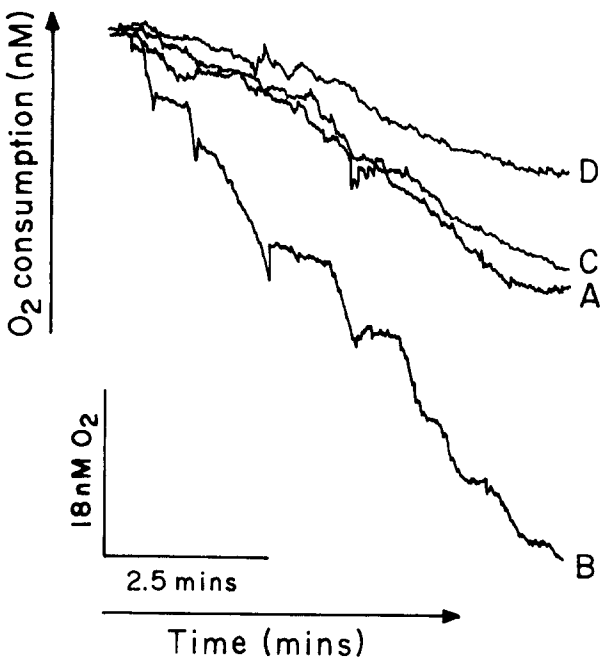

Fig. 3. Effect of MDC on IL-8 induced respiratory burst response in neutrophils. For this experiment, neutrophils were suspended in medium (2×10^6 cells in 2.2 ml) and incubated with MDC as before. In control and MDC treated cells, non-radioactive IL-8 (100 ng/ml) was added and incubated for 10 min at 37°C, and the oxygen consumption was recorded in the oxygraph. This figure shows the effect of MDC on the oxygen consumption of neutrophils in presence of IL-8 (100 ng/ml) recorded in a oxygraph. A: Control; B: IL-8 stimulated (100 ng/ml); C: dansyl cadaverine (900 μM) treated cells; D: dansyl cadaverine (900 μM)+IL-8 stimulated (100 ng/ml).

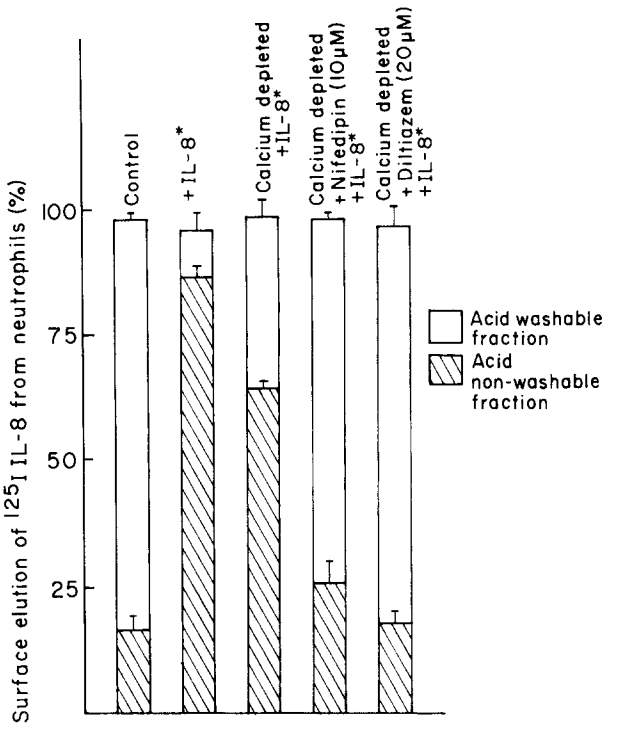

Fig. 4. IL-8 induced endocytosis in Ca^{2+} depleted condition in human neutrophils. Human neutrophils (2×10^6/tube) suspended in HEPES saline buffer (pH 7.2, 20 mM containing 0.5% BSA and 1 mg/ml glucose) were incubated with EGTA (1 mM), A23187 (20 μM), Quin 2AM (30 μM) for 15 min at 37°C. In other two sets, nifedipine (10 μM) and diltizem (20 μM) were also added separately. Two control sets, were run parallely. This figure presents the data obtained from the measurement of [^{125}I]IL-8 in cpm obtained from the acid washable and acid non-washable fractions of the tubes ± S.D. of duplicate samples.

3.5. Role of Ca^{2+}

Since Ca^{2+} is known to activate the enzyme-transglutaminase required for the process of endocytosis, we tested the role of Ca^{2+} in the IL-8 mediated endocytosis of its receptor.

Calcium depleted conditions for the study were developed by incubating the cells with EGTA (1 mM), A 23187 (20 μM) and Quin 2 AM (30 μM) for 30 min at 37°C in two sets and incubated separately with nifedipine (10 μM) and diltiazem (20 μM) for an additional 15 min. Then [^{125}I]IL-8 was allowed to bind to the cells at 4°C. After washing, all the cells except the control were warmed at 37°C for 25 min. The cells were then briefly exposed to acidic glycine solution to measure acid washable and non-washable fractions of [^{125}I]IL-8.

Data presented in Fig. 4 show that acid washable radioactivity in that from Control-I (4°C) and Control II (37°C) were 81% and 91%, respectively, whereas under Ca^{2+} depleted condition it was 34%. In nifedipine and diltiazem treated cells the acid washable fractions were 72% and 81%, respectively.

Direct effect of $CaCl_2$ on endocytosis was studied by developing a Ca^{2+} depleted condition using nifedipine and diltiazem as before. In one set with nifedipine and diltiazem, 1 mM $CaCl_2$ was also added. Now, IL-8 (500 ng/ml) was added to all the samples except Control-I and incubated at 4°C for 2 h. After washing, the cells were incubated at 37°C for 25 min. Then the cells were cooled, washed with acidic glycine solution to remove

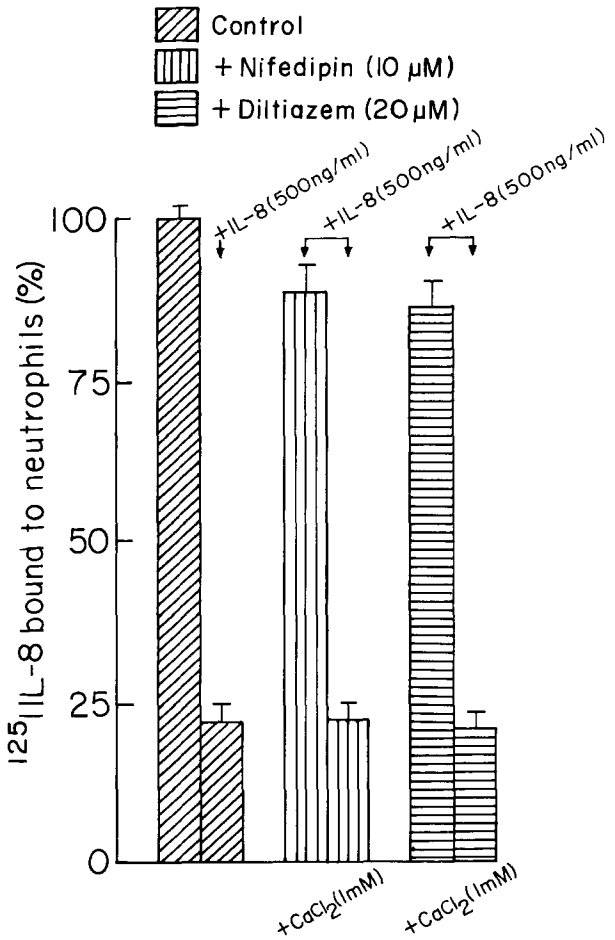

Fig. 5. Effect of Ca^{2+} on internalization of IL-8 receptors on human neutrophils. The cells suspended in 20 mM HEPES-saline buffer, pH 7.2 containing 0.5% BSA and 5 mg/ml glucose were incubated with EGTA, A23187 and Quin 2 AM as before. In two of the sets, nifedipine (10 μM) and in other two sets diltiazem (20 μM) were added and incubated for 30 min at 37°C. Two control sets were also run parallely. In one of the sets with nifedipin and diltiazem, 1 mM $CaCl_2$ was added. Then except Control-I, all the cells were treated with 500 ng/ml IL-8 (non radioactive) and incubated for 25 min at 37°C. After incubation, the cells were washed with acidic glycine solution and the total surface binding of [^{125}I]IL-8 to the cells were examined at 4°C. This figure shows [^{125}I]IL-8 binding to the cells in percentage ± S.D. of duplicate samples.

the unlabelled IL-8, and [^{125}I]IL-8 binding ability of the cells was examined at 4°C. The data presented in Fig. 5 demonstrate that with respect to Control-I (unlabelled IL-8 not added), Control II shows only 21.9% binding. The binding was 72% and 83%, respectively, with cells treated with nifedipine and diltiazem, under calcium depleted conditions. Presence of 1 mM $CaCl_2$ reduced the IL-8 binding of the nifedipine and diltiazem treated cells to 71.7% and 20.6% respectively.

4. Discussion

In this study we have presented some biochemical evidence in support of dansyl cadaverine mediated inhibition of IL-8 receptor endocytosis in human neutrophils.

Since cold acidic glycine solution at low pH can disrupt the non-covalent association of the receptor and the ligand, measurement of radiolabelled ligand in the acid washable and non-washable fractions provides a good method for measuring the amount of IL-8 present on the cell surface or inside the cell under different situations. In control, non treatment of cold IL-8 allowed full binding of radiolabelled IL-8 to the surface receptor as indicated in maximum availability of acid washable fraction of IL-8. In contrast, at 37°C the cell surface IL-8 receptors were rapidly internalized with the unlabelled ligand leaving only a small fraction of [^{125}I]IL-8 available in acid washable fraction and maximum was obtained in the acid non-washable fractions. In MDC treated cells [^{125}I]IL-8 content in acid washable fraction was considerably increased at 37°C, indicating that the receptors remain expressed on the cell surface due to inhibition of ligand induced internalization (detailed data not shown). This was further supported by comparing the kinetic analysis of the acid washable fractions of control and MDC treated cells where the second one shows maximum acid washable radiolabelled IL-8 at 37°C (Fig. 1). Thus it demonstrates that MDC somehow inhibits IL-8 mediated endocytosis of the receptor.

Again, at 37°C in presence of unlabelled IL-8, most of the IL-8 receptors are internalized with respect to control (IL-8 untreated), leaving only a small number of receptors on the cell surface detected by anti-IL-8R antibody and IgG. As MDC cannot induce Fc receptor expression in human neutrophils, increased [^{125}I]IgG binding in MDC treated cells must be due to increased binding of anti IL-8R antibody to the surface bound IL-8R. High binding of radiolabelled IgG to the MDC treated cells indicates the presence of a maximum number of IL-8R at 37°C even in the presence of the ligand. Therefore, it strongly corroborates that MDC indeed inhibits IL-8 induced internalization of the receptor.

Data presented here showed that in presence of MDC, the surface bound IL-8 was considerably higher than in control and also IL-8 induced migration of the cells was reduced. This can be explained only by assuming that MDC does not inhibit the binding of IL-8 to the cell surface but inhibits the signal transmission required for the migration of the cells. Inhibition of ligand induced endocytosis of the receptor is the most reasonable mode of action of MDC.

Respiratory burst response is an important property of actively phagacytosing neutrophils [19,20]. Since MDC has no effect on the metabolic activity of neutrophils, the oxygen consumption in control and MDC treated cells remained almost the same (Fig. 5). Oxygen consumption in IL-8 stimulated cells differed significantly in control and MDC treated cells. It indicates that although IL-8 strongly binds to the receptor, it fails to elicit an IL-8 mediated response supporting further the necessity of receptor mediated internalization of the cytokine for its biological response (Fig. 3).

Dansyl cadaverine is reported to be a potent inhibitor of serum and liver transglutaminase enzyme and in case of many polypeptides, hormones and growth factors, the receptor mediated endocytosis has been blocked by inhibiting the enzyme activity of transglutaminase [13,20]. Although we have not directly demonstrated the participation of the enzyme in the IL-8 induced endocytosis, a similar mechanism of blocking endocytosis can be envisaged from the selective action of dansyl cadaverine on the cell.

Under Ca^{2+} depleted conditions, when both extracellular and

intracellular free Ca^{2+} is chelated, perhaps, the transglutaminase becomes non-functional and IL-8 induced endocytosis is significantly reduced, leading to the presence of a large amount of labelled IL-8 in the acid washable fraction of the cells treated with nifedipine and diltiazem.

The inhibition of endocytosis observed under Ca^{2+} depleted conditions could be reversed by adding Ca^{2+}. In fact the extent of endocytosis in the cells which showed very low endocytosis during Ca^{2+} depleted condition in presence of nifedipine and diltiazem recovered to that in control in presence of 1 mM free $CaCl_2$ (Fig. 5). The reasons for absolute requirement of Ca^{2+} in the process have not been further explored. It has been demostrated that Ca^{2+} is essential for the Cu^{2+} mediated inactivation of transglutaminase [21]. There is substantial evidence that the enzyme cross-links the ligand and the receptor by catalysing a Ca^{2+} dependent acyl transfer reaction involving the gamma-carboxamide group of the glutamine residue(s) of the ligand. It seems that deficiency of Ca^{2+} impairs the catalytic activity of the enzyme and the cross linking of the ligand–receptor complex is prevented leading to inhibition of endocytosis.

Since IL-8 is an inflammatory cytokine, it is important to study the interaction of IL-8 with its receptor in target cells. We believe that the inhibition of IL-8 induced receptor internalization reported here will be helpful in exploring the intracellular signal transduction pathway. Identification of specific sequences in the cytoplasmic domains of membrane proteins that mediate rapid internalization through clathrin coated pits is an area of intense current research [22]. MDC may also be useful for identifying the particular motif in the IL-8 receptor that is involved in the internalization of the ligand. Dansyl cadaverine has been used to inhibit the entry of vesicular stomatitis virus, Semliki Forest virus and other viruses into host cells [21–22]. Replication and intercellular spreading of *Ehrlichia risticii* can also be almost completely prevented by the same agent [23]. Therefore, dansyl cadaverine may be a useful agent for blocking IL-8 induced excessive migration and accumulation of neutrophils during severe inflammatory distress.

Investigation of the effect of anti-inflammatory drug on IL-8 receptor–ligand interaction has been hampered by the rapid ligand induced endocytosis of the receptor at 37°C. Study of such interactions in presence of MDC may be a good model for in vitro study of anti-inflammatory agents.

In brief, the use of dansyl cadaverine for inhibiting IL-8 induced endocytosis of its receptor in human neutrophils reported here, may have potential significance for regulation of IL-8 induced inflammatory responses.

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