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Functions of interleukin-8 are mediated through thiol group(s) of IL-8 receptor in human polymorphonuclear neutrophils

Effects of 5,5′-dithio-bis(2-nitrobenzoic acid) on IL-8 receptor

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Interleukin-8, a neutrophil chemotactic agent causes excessive accumulation of the cells in a number of inflammatory diseases. The activity has been shown to be mediated through a specific functional receptor present on the surface of neutrophils. No information is available about the amino acids constituting the IL-8 binding domain of the receptor. Treatment of neutrophils with 5,5′-dithio-bis(2-nitrobenzoic acid), a thiol-specific modifier, at the concentrations of 0.4 mM and 1 mM reduced IL-8 binding ability and IL-8-induced migration of the cells by 45% and 65%, respectively. Dithiothreitol could regenerate the binding capacity and the ligand could protect the receptor from the effect of the reagent. All the evidence suggests that one or more critical thiol residues are located in the IL-8 binding site of the receptor which are indispensible for normal functions of IL-8.

IL-8 receptor; Neutrophil chemotaxis; IL-8 binding domain; Thiol residue

1. INTRODUCTION

Polymorphonuclear neutrophils (PMNs) provide a first line of defence against bacterial and fungal infection and play a vital role in host immunity. In non-infectious inflammatory diseases, the PMNs are excessively accumulated at the involved sites and augment pathological symptoms and prevent healing [1]. Interleukin-8 (IL-8), a monocyte-derived neutrophil chemotactic agent has been identified in a number of inflammatory diseases including psoriasis, rheumatoid arthritis and lung diseases. Its involvement in other inflammatory diseases has also been postulated [2–4]. As IL-8 is an important proinflammatory cytokine and it induces accumulation of PMNs resulting in augmentation of inflammatory responses, its mode of action on PMNs needs to be explored in detail.

A specific receptor for IL-8 has been identified on the surface of human PMNs and the role of the receptor in IL-8-mediated chemotaxis has been demonstrated [5,6]. The amino acid sequence encoded by the cDNA clone of IL-8 receptor shows that the receptor belongs to the superfamily of G-protein-linked receptors that contain seven transmembrane domains [7,8]. Two forms of the IL-8 receptor are known. One form has high affinity for IL-8 itself and two agonists, namely MGSA (GROα) and NAP-2. The other form of receptor shows low affinity for NAP-2 and MGSA but high affinity for IL-8 [9].

Although the importance of the IL-8 receptor during IL-8-induced chemotaxis has been demonstrated, the mode of interaction of IL-8 with its receptor remains obscure. Almost nothing is known about the composition and architecture of the ligand-binding domain of the receptor.

5,5′-Dithio-bis(2-nitrobenzoic acid), a mild thiol-specific modifier originally introduced by Ellman is a widely used reagent for location and quantitative determination of free -SH groups in proteins as well as in various biological fluids and tissues [10,11]. Using the reagent in the present study, we have shown that the ligand-binding ability as well as other functions of the IL-8 receptor can be disrupted by treatment with 5,5′-dithio-bis(2-nitrobenzoic acid) which were reversibly restored by DTT, suggesting the presence of active thiol residue(s) in the binding site of IL-8 receptor.

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 the Dainippon Pharmaceutical Company, Osaka, Japan. Dithiothreitol, 5,5′-dithio-bis(2-nitrobenzoic acid), nitroblue tetrazolium were from Sigma Chemical Co., St. Louis, MO, USA. Ficoll Paque, Sephadex G-10, Dextran T-500 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

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The PMNs were separated from fresh human peripheral venous blood of a normal healthy donor by dextran sedimentation followed by the Ficoll–Paque centrifugation method [12]. The preparation contained 90–95% PMNs of which 98% were viable.

Human recombinant IL-8 (10 μg) was labeled with ^{125}I using Bolton Hunter reagent [13]. The specific activity of the labeled material was 1×10^7 cpm/μg protein. The biological activity of IL-8 was examined in a modified Boyden chemotactic chamber (Neuro probe, Inc., Bethesda, MD), using a polyvinyl pyrrolidone-free polycarbonate filter (10 μm thick, 3 μm pore size [6,14]).

2.1. *Enzyme release assay*

For this assay 2×10^6 cells/200 μl PBS (pH 7.7) were incubated with DTNB for 30 min at 37°C. Reagent-free PMNs were suspended in D-PBS and treated with cytochalasin B (5 μg/ml) for 5 min. The PMNs were incubated with 100 ng/ml IL-8 for 45 min at 37°C. Myeloperoxidase activity was measured in 100 μl of supernatant using citrate–phosphate buffer (0.1 M, pH 5.2, 100 μl) containing 0.01% H_2O_2 and 1 mM tetramethyl benzidine. After stopping the reaction with 50 μl $4(N)H_2SO_4$, the absorbance was measured at 492 nm [15,16].

2.2. *NBT test*

For the test PMNs (1×10^7 cells/ml) suspended in PBS (pH 7.7) were treated with DTNB and incubated for 30 min at 37°C. After removing the excess reagents, the functional activity of PMNs was determined by NBT test where the capability of the cells to reduce the yellow-coloured dye to a deep blue product, formazan, was observed. The NBT-positive cells were counted under a phase-contrast microscope [17,18].

3. RESULTS

As we wanted to examine the functional role of amino acid residues constituting the binding domain of IL-8 receptor in responding cells, we used intact human PMNs for our experiments instead of purified IL-8 receptor. The PMNs were treated with DTNB, *N*-ethylmaleimide and diamide, excess reagents were removed and the binding ability of the cells towards ^{125}I-IL-8 was measured and biological responses of the modified cells (e.g. receptor–ligand internalization and recycling of the receptor, chemotaxis, enzyme release) were evaluated and compared with the control.

3.1. *Binding inhibition*

Preincubation of PMNs with cell non-penetrating thiol reagent DTNB at 37°C resulted in loss of ^{125}I-IL-8 binding capacity in a dose-dependent manner. Thus, addition of 0.4 mM and 1 mM reagent to the incubation mixture gave 25% and 62% loss, respectively, of binding capacity at pH 7.3. The inhibition was increased to 45% and 68%, respectively, at the same concentration of reagents when the pH of the incubation mixture was raised to 7.7 (Fig. 1). In a separate experiment, treatment with cell penetrating thiol reagents *N*-ethylmaleimide (1 mM) and diamide (0.4 mM) was shown to cause 60–65% inhibition of binding of ^{125}I-IL-8 to PMNs. This suggests that the PMNs are affected by both the cell non-penetrating and cell penetrating thiol modifying reagents and that the DTNB reaction is pH dependent (Fig. 1). Further experiments with DTNB were carried out at pH 7.7, because of higher inhibition at this pH.

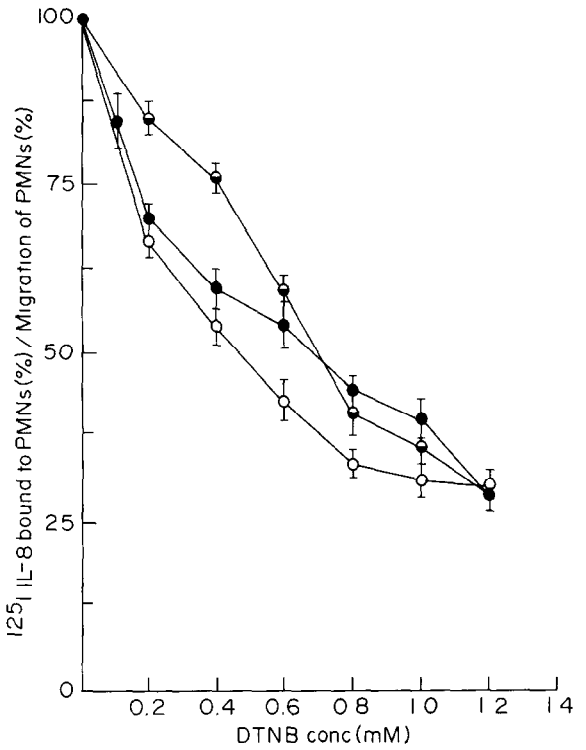

Fig. 1. Effect of DTNB concentration used on the binding of ^{125}I-IL-8 to and IL-8 induced migration of neutrophils. Human neutrophils (2×10^6 cells/200 μl suspended separately in PBS (pH 7.3 and 7.7) containing 1 mg/ml D-glucose) were incubated for 30 min at 37°C with various amounts of DTNB as indicated in the figure. After incubation the excess reagent was removed and the cells were suspended in binding medium (D-PBS containing 10 mg/ml BSA and 1 mg/ml glucose) and incubated for 2 h at 4°C with ^{125}I-IL-8 (4 ng/tube). The labeled IL-8 bound to neutrophils was measured. The results shown are the mean cpm ± S.D. of duplicate measurements. Binding capacity at pH 7.3 (◒); binding at pH 7.7 (○); migration of PMNs at pH 7.7 (●). For chemotaxis experiments neutrophils (1×10^6 cells/ml) were treated with same doses of DTNB used for binding experiments and after removing excess reagents the cells were suspended in binding medium. In the lower chamber of a Boyden chemotactic chamber 26 μl of 50 ng/ml IL-8 was added and in the upper chamber 50 μl of reagent-treated neutrophils (50,000/well) was applied in each well. As a reference control fMLP (1×10^{-8} M) was used. The chamber was incubated in a 5% CO_2 incubator containing humidified air. After the incubation the membrane was removed, fixed with 70% methanol and stained with Giemsa and the migrated cells were counted under a phase contrast microscope. The mean count of cells from different position of each spotted area was calculated. The figure represents the percent mean ± S.D. values of IL-8-induced migration of neutrophils in the presence of DTNB in duplicate samples.

3.2. *Regeneration of IL-8 binding capacity*

We attempted to reverse the DTNB-mediated loss of IL-8 binding ability of PMNs and we observed that DTT could restore the binding capacity of DTNB-treated PMNs. The binding capacity of PMNs which were reduced to 53%, 40% and 35% by treatment with 0.4 mM, 0.8 mM and 1.2 mM DTNB were increased to 87%, 80%, and 76%, respectively, on treatment with 2 mM DTT. Incubation with DTT alone showed a 5–10%

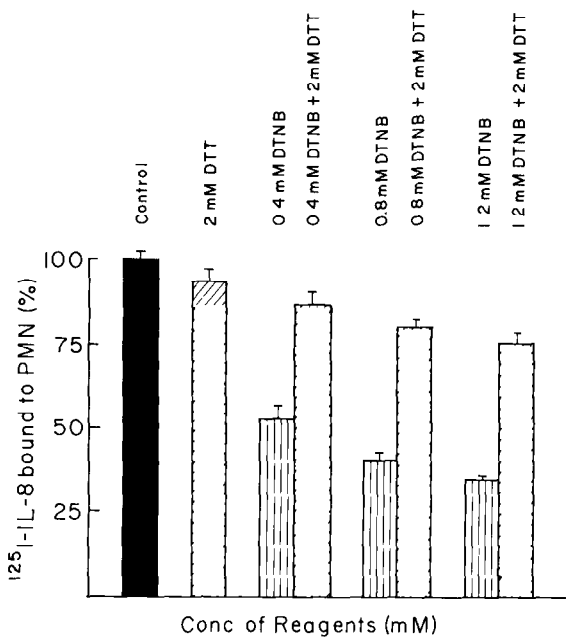

Fig. 2. Regeneration of IL-8 binding capacity of DTNB-inactivated PMNs by dithiothreitol. Human PMNs (2×10^6/200 μl) suspended in PBS (pH 7.7) were treated with the indicated doses of DTNB and incubated for 30 min at 37°C. After removal of excess reagents the cells were suspended in D-PBS (pH 7.2) and 2 mM dithiothreitol was added and further incubated for 15 min at 37°C. By removing excess dithiothreitol the binding ability of neutrophils was measured as in Fig. 1. The data represents the percentage of radiolabeled IL-8 bound to the neutrophils obtained from the mean cpm ± S.D of duplicate measurements.

decrease of IL-8 binding of the control value (Fig. 2). Thus the DTNB mediated modification of -SH groups of IL-8 receptors were reversibly regenerated by DTT.

3.3. *Ligand protection*

To find out whether the DTNB-modified thiol groups belong to the IL-8 binding site of the receptor or are derived from other proteins, we carried out a ligand protection experiment. As can be seen from the data presented in Table I, prior incubation of the cells with the ligand ensured significant protection against DTNB induced inactivation of PMNs at three concentrations of the modifier.

3.4. *Effect of total uptake of IL-8*

Incubation of PMNs with IL-8 at 37°C can be used to check the functional aspects of the receptor, which include binding of the ligand, internalization, separation of receptor from ligands and translocation of the receptor to re-express on the cell surface [6]. We examined these functions of the receptor in PMNs with and without treatment with DTNB. The results, presented in Fig. 3, demonstrate that total uptake of IL-8 was increased with the dose of IL-8 present in the incubation mixture. The uptake was reduced to about 50% in DTNB treated PMNs. Under identical conditions *N*-ethylmaleimide showed markedly reduced uptake of the ligand (10–20%).

3.5. *Effect on chemotaxis*

Directed migration of PMN is a good index of functional viability of cells and IL-8-induced chemotaxis can be used for this purpose. In the present study, PMNs were treated with DTNB and then the IL-8-induced chemotactic response of PMNs was examined in a Boyden chamber. As is seen in Fig. 1, DTNB-treated cells showed inhibition of migration in a dose-dependent manner. The reagent-treated cells at concentrations of 0.1 mM, 0.4 mM and 1.2 mM, showed 15%, 40% and 70% inhibition of migration, respectively. Under identical conditions, 0.1 mM and 0.4 mM *N*-ethylmaleimide could block PMN migration by 60% and 96%, respectively.

3.6. *Effect on enzyme release*

Degranulation of the lysosomal enzymes is induced by chemoattractant and such enzymes can be quantitatively measured in cytocholasin-B-treated cells. This function is also mediated by the receptor and is a good indication of chemoattractant-mediated cell activation. DTNB-treated PMNs, when stimulated by IL-8, released myeloperoxidase enzyme in a dose-dependent manner. For example, cells treated with 0.4 mM, 0.8 mM and 1.2 mM DTNB when stimulated with 100 ng/ml of IL-8 showed 76%, 61% and 56% myeloperoxi-

Table I

Protection of PMNs by ligand from the effect of DTNB

Reagent(s) added to PMNs	^{125}I-IL-8 bound to PMNs (cpm)
1. Control, acid wash + ^{125}I-IL-8	8,839 ± 372
2. IL-8 (0.5 μg) + ^{125}I-IL-8	527 ± 120
3. DTNB (0.4 mM), acid wash + ^{125}I-IL-8	5,921 ± 216
4. IL-8 (0.5 μg) + DTNB (0.4 mM), acid wash + ^{125}I-IL-8	8,542 ± 332
5. DTNB (0.8 mM), acid wash + ^{125}I-IL-8	5,172 ± 226
6. IL-8 (0.5 μg) + DTNB (0.8 mM), acid wash + ^{125}I-IL-8	7,622 ± 296
7. DTNB (1.2 mM), acid wash + ^{125}I-IL-8	3,453 ± 236
8. IL-8 (0.5 μg) + DTNB (1.2 mM), acid wash + ^{125}I-IL-8	6,315 ± 321

For protection experiment, human PMNs (2×10^6 cells/200 μl PBS, pH 7.7 containing 1 mg/ml D-glucose) were incubated with and without 0.5 μg unlabeled IL-8/tube for 2 h at 4°C. Different concentrations of DTNB as indicated in the Table were added and further incubated for 1 h at 4°C. The cells were washed with D-PBS and except for one pair (entry 2) the pellets were treated with cold glycine-HCl (pH 3.0, 50 mM)/NaCl (0.1 M) mixture for 60 s to remove unlabeled IL-8 bound to PMNs. The acid-washed cells were suspended in 'binding medium' (D-PBS containing 10 mg/ml BSA, 1 mg/ml glucose) and 4 ng (40,000 cpm) of ^{125}I-IL-8 was added per tube. The data represent the mean counts per minute ± S.D. of radiolabeled IL-8 bound to PMNs in duplicate samples

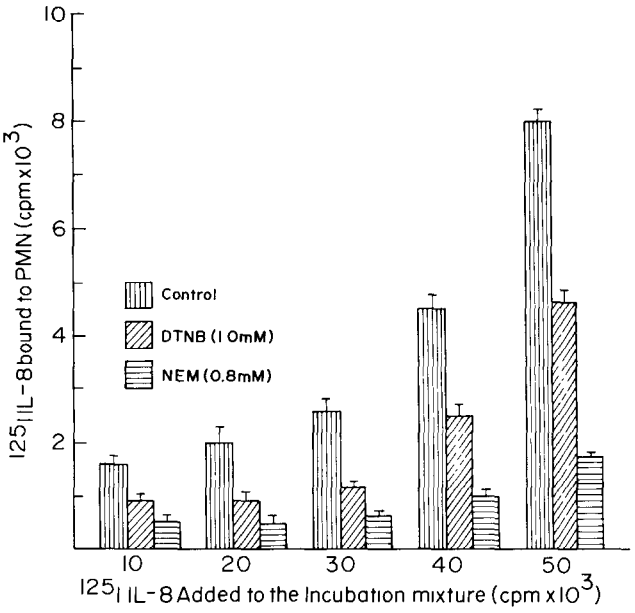

Fig. 3. Effect of DTNB and NEM on the total uptake of IL-8 to PMNs at 37°C. For this experiment, freshly prepared human neutrophils (2×10^6 cells/200 μl) suspended in PBS (pH 7.7) containing 1 mg/ml D-glucose were treated with 1 mM DTNB and 0.8 mM NEM separately and incubated for 30 min at 37°C. After removing the excess reagents, the cells were suspended in D-PBS and cultured for 1 h at 37°C in the presence of various amounts of ^{125}I-IL-8 as indicated in the figure. The bound activity (total uptake of IL-8) to the neutrophils was then measured. The result shows mean cpm ± S.D. of duplicate samples of a representative experiment.

dase activity, respectively. Under identical conditions, 0.4 mM and 0.8 mM *N*-ethylmaleimide could release 50% and 38% enzyme, respectively.

3.7. *Functional tests*

The thiol reagents may be cytotoxic to the cells and may interfere with the general metabolic functions of the cells. For that reason, viability of the cells was examined using the Trypan blue dye exclusion test. The viability of the DTNB-treated cells was 83% and 72% when PMNs were treated with 0.2 mM and 0.8 mM DTNB, respectively. PMNs treated with the same strength of *N*-ethylmaleimide showed 85% and 79% viability, respectively. Reduction of NBT to coloured formazan by LPS stimulated PMNs reflect general metabolic activities of the cells. The DTNB treated cells were subjected to this test and the result demonstrates that compared to control, 0.2 and 0.8 mM of the reagents showed 88% and 78% NBT positive cells for DTNB suggesting that the cells are not severely damaged by the reagents.

4. DISCUSSION

Directed migration of cells during chemotaxis is initiated and sustained by the tight association of the chemoattractant to its receptor on the target cell. Chemical modification of the amino acids in the binding domain of the receptor may alter receptor–ligand interaction as well as receptor-mediated functions of the cell.

Interleukin-8 is secreted from the foci of injury during the onset of inflammation. Strong binding of IL-8 to its receptor on the PMNs, not only indicates the nature and direction of the source of the inflammatory stimulus but also induces activation of the contractile elements of PMNs for migration. In the present study, we have presented evidence which strongly indicates the participation of functionally critical sulfhydryl group(s) of the receptor. Covalent modification of these thiol groups severely impairs the binding of the ligand to the receptor as well as receptor-mediated functions.

5,5′-Dithio-bis(2-nitrobenzoic acid), introduced as mild thiol-specific reagent which converts free sulfhydryl groups of proteins to thionitrobenzoate groups with concomitant liberation of one equivalent of 5-thio-2-nitrobenzoate anion [10,11]. In our case treatment of PMNs with the reagent presumably reacts with the free -SH group(s) of cysteine residues in the IL-8 binding domain of the receptor resulting in similar thionitrobenzoate protein complex. The modification reduces the binding of the receptor to IL-8 as is evident from reduced binding of ^{125}I-IL-8 to PMNs (Fig. 1). DTNB is reported to be more reactive towards thiol groups at higher pH. We also obtained higher inhibition at pH 7.7 compared to pH 7.3 indicating more extensive modification of the SH groups at the higher pH.

Dithiothreitol added to the DTNB modified PMNS displaces the thionitrobenzoate groups restoring the free -SH groups of the receptor leading to regeneration of binding capacity of the receptors towards IL-8 (Fig. 2). Prior occupation of receptors by unlabeled IL-8 afforded significant protection of the receptor from the effects of DTNB. It suggests that -SH group(s) may be located at the binding site of IL-8 and thus escape modification. Alternatively, binding of IL-8 to the receptor may induce conformational changes in the receptor making some of the thiol groups inaccessible to the reagent (Table I).

The reagent, DTNB is known to be a cell non-penetrating thiol modifier and as such it could modify -SH groups of the surface receptors. After fresh culture of DTNB-pretreated PMNs at 37°C, the intracellular unmodified receptors were expressed on the cell surface and about 50% of total uptake of IL-8 was observed. In contrast *N*-ethylmaleimide, a cell penetrating thiol modifier, could modify both intracellular and extracellular receptors. For that reason culture of NEM-treated PMNs with IL-8 showed only 10–20% total uptake of IL-8 (Fig. 3). This also explains the nature of the dose-dependent variation of chemotaxis in DTNB-treated cells. As can be seen from Fig. 1, with increasing concentration of DTNB used, the inhibition of chemotaxis reached saturation well below 100% and about 30% residual chemotaxis was observed even at DTNB con-

centrations as high as 1.2 mM. This can be explained as being due to re-expression of intracellular and buried receptors unaffected by DTNB. In contrast, NEM-treated cells showed complete inhibition of chemotaxis at higher reagent concentrations. Similar difference in the levels of enzyme released in DTNB- and NEM-treated cells can be explained by the difference in the abilities of the two reagents to modify intracellular receptors.

The cytotoxicity of DTNB towards PMNs was examined by the NBT test which showed that the general metabolic activities of the cells remains significantly unaltered as evidenced from the examination of functional test of DTNB treated PMNs.

All the evidence discussed above strongly indicate the presence of DTNB-modifiable -SH groups at the ligand-binding domain of the IL-8 receptor. The cDNA encoding the IL-8 receptor from human PMNs shows the amino acid sequence of the receptor as having 10 cysteine residues. In the proposed model of the IL-8 receptor, 6 cysteine residues are embedded in the transmembrane regions and 4 cysteine residues are exposed in the extracellular region [7]. One or more of the extracellular cysteine residues may be susceptible to DTNB modification. Knowledge about the number of -SH groups involved and the mode of their participation in the ligand binding must await a detailed characterization of the receptor protein.

A large number of membrane receptors, e.g. rhodopsin and visual pigments, the family of muscarine receptors, adrenergic receptors and others belong to the superfamily of G-protein-linked receptors and have seven transmembrane helices like IL-8 receptor. The apparent similarities in structure and function among the members of this class of receptors indicate the possibility of a common underlying mechanism for the transduction of signal [19]. Rhodopsin has also three cysteine residues located in the extracellular domains of the secondary structure model. Two of the extracellular cysteines are essential for retinal binding, and by replacing cysteine with serine it has been shown that out of the 10 cysteine residues only these two are required for maintaining the correct tertiary structure of the protein [20]. To understand the mechanism of signal transduction in this class of receptors some insight into the structural interrelationship of the receptor domain is needed. In this respect the evidence presented here for involvement of cysteine residues in ligand binding of the IL-8 receptor may be useful in understanding the nature of the ligand–receptor interaction of other receptors of this family.

In summary, our studies provide information for one or more critical sulfhydryl groups in the IL-8 binding site of the receptor which seems to be indispensable for normal functioning of the receptor. As the -SH groups are critical for normal function, they may be a potential target for modulation of IL-8 activity and also for screening and designing of antiinflammatory drugs.

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