Upregulation of interleukin-8 receptor in human polymorphonuclear neutrophils by formyl peptide and lipopolysaccharide

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Abstract Interleukin-8 (IL-8) is implicated in the pathogenesis of a large number of neutrophil-driven inflammatory diseases. Although the cytokine activates neutrophils through a receptor, no information is available regarding the regulation of IL-8 receptor (IL-8R) expression. The present study shows that, compared to control, the bacterial products – formylpeptide and LPS (serum-activated) upregulate IL-8 receptor by 54% and 115%, respectively, the former by degranulation of the secretory vesicle and the latter by de novo protein synthesis. The newly expressed IL-8R could be demonstrated with anti-IL-8R-antibody and by autoradiogram of the receptor crosslinked with [^{125}I]IL-8. The study may be useful for understanding the potential role of IL-8 during neutrophil mediated inflammatory response.

Key words: Interleukin-8; IL-8 receptor; Lipopolysaccharide; fMLP; Inflammation

1. Introduction

Polymorphonuclear neutrophils (PMNS), the wandering phagocytic white blood cells provide a first line defense in host against bacterial and fungal infections. During bacterial infection, lipopolysaccharides, derived from the cell wall of Gram-negative bacteria stimulate the synthesis and secretion of interleukins in a number of cells. One such stimulatory effect of LPS in monocytes is the secretion of Interleukin-8 which can chemoattract polymorphonuclear neutrophils in vitro and in vivo [1–3]. Recently, IL-8 has been implicated in the pathogenesis of a large number of neutrophil driven inflammatory diseases like joint diseases, lung diseases and skin diseases [2–4]. The cytokine activates neutrophil and augments inflammatory responses by inducing release of free toxic oxygen radicals and proteolytic enzymes [3,4]. The function of IL-8 is mediated through a receptor expressed on the surface of neutrophils [5,6]. Two types of the glycosylated IL-8 receptors, termed A and B, are known to be expressed in the same mammalian cell types [7,8]. At 37°C, the receptors are rapidly downregulated with its own ligand IL-8 and then the internalised receptors are recycled back to cell surface to accept fresh ligands. Continuous recycling of the receptors is linked to the IL-8 induced migration of neutrophils [9]. The receptor belongs to the superfamily of seven transmembrane domain containing proteins that bind to G-protein [4,10]. Recently, it has been reported that, the N-terminal region and the extracellular loop-3 of the receptor lie in close proximity and together constitute the major binding domain for IL-8 [11]. We have demonstrated that thiol residues of the IL-8R participate in the ligand binding and their chemical modification impairs IL-8 mediated functions in neutrophils [12,13].

Although IL-8 has been implicated in the pathogenesis of a large number of neutrophil driven inflammatory diseases and its receptors play a crucial role in the IL-8 mediated functions, knowledge about the regulation of IL-8 receptor expression on the surface of target cells is meagre and needs investigation in detail. The bacterial product – fMLP is a potent stimulator of phagocytic cells causing respiratory burst response and release of lysosomal enzymes [14]. The LPS is also a potent stimulant which triggers cellular, physiological and metabolic activities of the host [15]. In this communication we present evidences that fMLP and LPS upregulate the IL-8R in human neutrophils.

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 Co., Osaka, Japan. *E. coli* LPS (055:B5), *N*-formyl-methionyl-leucyl-phenylalanine, cycloheximide, actinomycin D, colchicine, chloramine-T, polymixin B sulfate, calcium ionophore (A23187), anti-human CD14 MOAb (UCHM-1), anti-rabbit IgG antibody (raised in goat) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Ficoll-paque, Sephadex G-10 and Dextran T-500 were obtained from Pharmacia Fine Chemicals, Upsala, Sweden. Other chemicals and reagents were of analytical grade. 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.

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

Human recombinant IL-8 and anti-rabbit IgG antibody were radiolabelled with ^{125}I by chloramine-T following the method of Grob et al. [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 were examined in a modified Boyden chemotactic chamber (Neuroprobe Inc., Bethesda, MD), using polyvinyl pyrrolidone free polycarbonate filter (10 μm thick, 3 μm pore size) [9,12].

The chemical crosslinking was carried out following the method of Dower [18]. Human neutrophils were incubated with [^{125}I]IL-8 at 4°C. After washing with cold D-PBS the cells were suspended in 100 μl D-PBS. Then DSS (1 mg/ml) was added with gentle stirring and incubated at 4°C for 1 h. After washing the cells, 100 μl CHAPS (9 mM) in D-PBS and a cocktail of protease inhibitors containing PMSF (200 μM), leupeptin (1 μM), pepstatin (1 μM) and EDTA (100 μM) were added and kept for 10 min in ice. After mixing thoroughly, centrifugation was carried out at 4°C for 15 min at 10,000 rpm. The supernatant was analysed in SDS-PAGE (10%) under reducing condition. The gel was dried and autoradiography was carried out using Kodak X-Omat-K film at −70°C.

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3. Results

3.1. Effect of formyl peptide

Our primary interest was to examine whether fMLP had any effect on the IL-8 receptor level in PMNs. For that purpose, we incubated human PMNs for 30 min at 37°C with different amounts of fMLP and after washing, the binding of [^{125}I]IL-8 to PMNs was measured. As can be seen in Fig. 1, IL-8 binding to PMNs was increased upto 1 μM fMLP in a dose dependent manner which was reduced upon further addition of fMLP. An optimum dose of fMLP (1 μM) could elicit a maximum of 54% increase ($P < 0.001$) in the binding of IL-8 to PMNs with respect to control (Fig. 1).

3.2. Source of IL-8R

To find out the source of IL-8R two metabolic blockers – actinomycin D (1 μM) and cycloheximide (10 μg/ml) were preincubated with PMN separately before addition of fMLP. Neither of the compounds had any significant effect on IL-8 binding. Treatment with colchicine (50 μM), an inhibitor of translocation of granules by the microtubular system, diminished the fMLP induced IL-8R expression by about 95%, indicating that the intracellular granules may be possible source of IL-8R.

To ascertain the source, the activity of the marker enzymes of the granules of PMNs – myeloperoxidase (azurophilic), lysozyme (specific), alkaline phosphatase (secretory vesicles) were carefully measured in the cultured supernatants after fMLP treatment. The alkaline phosphatase activity of the supernatant was found to increase with fMLP concentration used and correlated with the increase in IL-8 binding (Fig. 1). Treatment with colchicine and subsequent stimulation with fMLP, reduced both the binding of IL-8 to PMNs and the alkaline phosphatase level in the cultured supernatant (Fig. 1). The levels of myeloperoxidase and lysozyme in the cultured supernatant were not altered by fMLP. We did not directly measure the level of gelatinase, a marker enzyme for gelatinase granules. It has been reported that fMLP at doses of 0.01–1 μM increased the release of gelatinase by 25–30%. But we have studied the effect of A23187, a calcium ionophore, which at the doses of 0.3 μM and 1 μM were reported to induce release of gelatinase by 25% and 74%, respectively [19]. Under identical condition we could not find any increased IL-8 binding on the surface of neutrophil over the control level (data not presented).

3.3. Effect of LPS

The effect of bacterial LPS on IL-8R expression, was studied by incubating PMNs with 1 ng to 1 μg/ml LPS for 30 min at 37°C. Only 10–18% IL-8 binding was increased with respect to control (Fig. 2).

Then the LPS was incubated with autologous serum for 1 h at 37°C. With this LPS the PMNs were incubated for 30 min at 37°C, and the binding of IL-8 to PMNs was increased by 115% ($P < 0.001$) and 94% ($P < 0.001$) over the control at the conc. of 10 ng and 100 ng/ml serum activated LPS (SA-LPS), respectively. In the subsequent experiments, 10 ng/ml SA-LPS, which was the optimum dose for the maximum binding of IL-8 was used (Fig. 2).

3.4. Source of IL-8R

Actinomycin D and cycloheximide, the metabolic blockers, were ineffective in blocking LPS mediated stimulation but as in the case of fMLP induced stimulation, 90% of IL-8 binding and alkaline phosphatase release were inhibited by colchicine (50 μM). The results were opposite during SA-LPS mediated stimulation where colchicine was ineffective in reducing IL-8 binding, and no significant release of alkaline phosphatase was detected. But the IL-8 binding was strongly inhibited by both actinomycin D (1 μM) and cycloheximide (10 μg/ml). Under identical conditions, incubation of control cells with the metabolic blockers did not show any alteration of IL-8 binding.

3.5. Effect of polymixin B and anti-CD14 antibody

Poymixin B is known to interact with LPS and may neutralize the effect of LPS. To examine this, polymixin B (50 μg/ml) was added during incubation of LPS with serum at 37°C and with this LPS no significant increase in binding of the IL-8 to PMN surface was observed. A receptor for LPS, CD14, is moderately expressed on the surface of PMNs and may participate during SA-LPS mediated stimulation. In order to investigate that possibility, anti-CD14-antibody (mAb) was treated with PMNs and stimulation with LPS (serum activated) showed 94% inhibition of IL-8 binding with respect to control (Fig. 2).

3.6. Anti-IL-8R antibody

Increased binding of IL-8 in fMLP and SA-LPS stimulated neutrophils were due to induction of IL-8R expression, can be

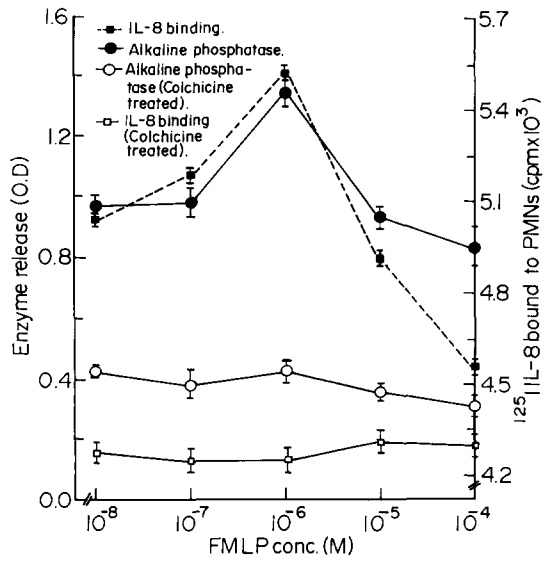

Fig. 1. Effect of various doses of fMLP on the binding [^{125}I]IL-8 to neutrophils and release of alkaline phosphatase. Human neutrophils (2×10^6 cells/200 μl) suspended in D-PBS containing 1 mg/ml BSA were preincubated with and without colchicine (50 μM) at 37°C for 30 min. Then various amounts of fMLP as indicated in the figure were added to the incubation mixture and the incubation was continued for 30 min at 37°C. After incubation the cells were suspended in binding medium (RPMI-1640 containing 10 mg/ml BSA and 20 mM HEPES buffer) and incubated at 4°C for 2 h with [^{125}I]IL-8 (4 ng/tube). After washing the radiolabelled IL-8 bound to neutrophils was measured in a gamma counter. The results shown are the mean cpm ± S.D. of duplicate measurements. For the assay of alkaline phosphatase, 25 μl of the supernatant was taken from the incubation mixture of the above experiment and mixed with 50 μl of 1.0 M ethanolamine, pH 9.8, containing 0.5 mM $MgCl_2$, 15 mM *p*-nitrophenyl phosphate and incubated at 37°C for 30 min and the absorbance measured at 405 nm. The results plotted are the mean ± S.D. of triplicate assay.

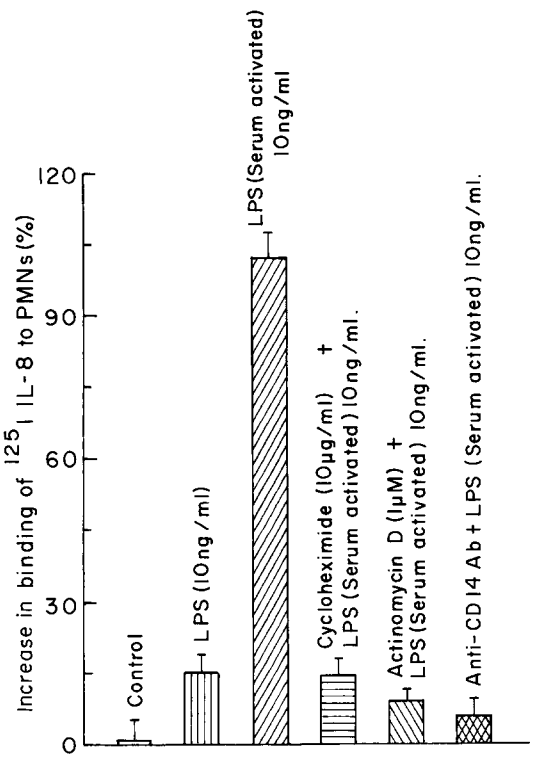

Fig. 2. Effect of metabolic inhibitors and anti-CD14 antibody on binding of IL-8 to the LPS (serum activated) stimulated neutrophils. Human neutrophils (2×10^6 cells/200 μl) suspended RPMI-1640 containing 10 mg/ml BSA and 20 mM HEPES buffer, pH 7.2, were incubated 37°C in three sets of tubes. In one set of tubes anti-CD14 antibody (4 μl) was added to the cells and incubated for 15 min. Another two sets were incubated separately with cycloheximide (10 μg/ml) and actinomycin D (1 μM) for 30 min under the same conditions. Serum activated LPS (10 ng/ml) was added to all the tubes and incubated at 37°C for 30 min. Three controls were run simultaneously in presence of no LPS, 10 ng/ml serum free LPS and 10 ng/ml SA-LPS but without the antibody or the drugs. After incubation, the cells were washed with fresh medium, cooled and binding were examined using 4 ng IL-8/tube. The figure presents percentage increase in binding of [^{125}I]IL-8 to the surface of PMNs ± S.D.

verified by estimating the level of IL-8 receptor protein on the cell surface. For this purpose, we incubated unstimulated PMNs and as well as fMLP and SA-LPS stimulated PMNs with anti-IL-8R antibody. After washing the first antibody, we incubated the cells with ^{125}I-labelled second antibody (anti-rabbit IgG) for 1 h at 37°C. Then the total binding of IgG was determined in PMNs. As was to be expected, the binding of IgG in fMLP and LPS stimulated neutrophils were increased by 75% ($P < 0.001$) and 121% ($P < 0.001$), respectively, over the unstimulated cells (Fig. 3). Addition of second antibody to the fMLP and SA-LPS stimulated cells showed no increase in binding of the labelled IgG with respect to control (data not presented).

3.7. *Scatchard analysis*

The Scatchard analysis was carried out in unstimulated, fMLP and SA-LPS stimulated PMNs with saturable amounts of IL-8 at 4°C. The Scatchard plot indicates that all the receptors belong to single class (Fig. 4). The receptor numbers for unstimulated, fMLP and SA-LPS stimulated PMNs were 23,000, 39,000 and 47,000 per neutrophil and the K_d values for these receptors were 2.7 nM, 3.4 nM and 3.7 nM, respectively (Fig. 4).

3.8. *Affinity crosslinking*

In order to demonstrate the increased number of IL-8R on PMN surface, the chemical crosslinking of IL-8 to the receptor was carried out at 4°C by a bifunctional crosslinking agent, DSS, in unstimulated, fMLP and SA-LPS stimulated PMNs. Fig. 5 presents the autoradiogram obtained after SDS-PAGE analysis under reducing condition of the proteins extracted with the detergent (CHAPS; 9 mM) from the crosslinked cells. Compared to control, the intensity of the radioactive band at 67 kDa was increased in the fMLP and SA-LPS stimulated cells. The signal from SA-LPS stimulated PMNs was significantly reduced in the presence of excess unlabelled IL-8 (Fig. 5, lane 4).

4. Discussion

In this communication, we have presented evidences in support of bacterial products – formyl peptide and lipopolysaccharide mediated upregulation of IL-8 receptor on the surface of human neutrophils. The formyl peptide has been reported to activate PMNs through a specific cell surface receptor and triggers the inflammatory response [14]. The fMLP induced

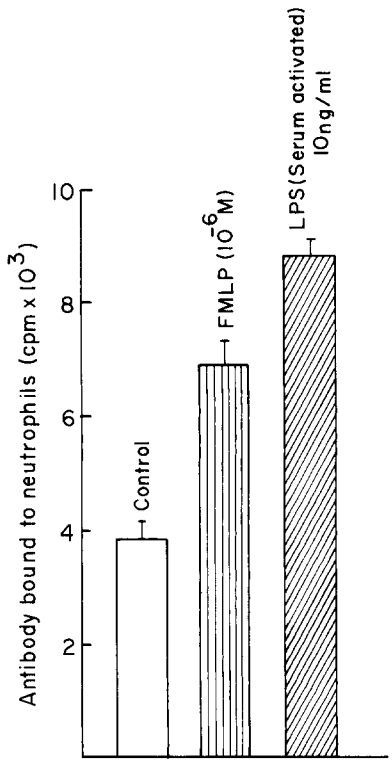

Fig. 3. Effect of anti-IL-8 receptor antibody on the fMLP and SA-LPS stimulated neutrophil. Human neutrophils (2×10^6 cells/200 μl) were suspended in RPMI-1640 containing 10 mg/ml BSA and HEPES buffer, pH 7.2. In each tube anti-IL-8 receptor antibody (1:100 diluted) was added and incubated at 37°C for 45 min. The cells were washed, suspended in RPMI-1640 and finally incubated with ^{125}I-labelled anti-rabbit IgG at 37°C for 1 h. The binding of second antibody was measured in a gamma-counter. The figure demonstrates the specific binding of [^{125}I]IgG to the unstimulated, fMLP and SA-LPS stimulated PMNs in cpm ± S.D. in duplicate samples.

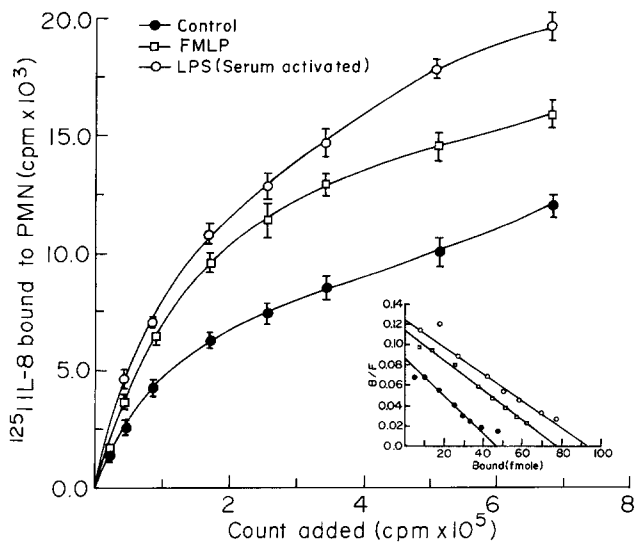

Fig. 4. Scatchard analysis of control, fMLP and SA-LPS stimulated neutrophils. Neutrophils, suspended in binding medium were incubated with fMLP (10^{-6} M) and SA-LPS (10 ng/ml) at 37°C for 30 min. Then the cells were incubated at 4°C for 2 h with various amounts of [^{125}I]IL-8 as indicated in the Fig. 4. Another set, without LPS was run in parallel. To determine specific binding, the non-specific bindings (obtained from experiments done in presence of 100-fold excess cold IL-8) was subtracted. The result shown is representative of three independent experiments. (●) Neutrophils (control); (□) neutrophils (fMLP stimulated); (○) neutrophils (SA-LPS stimulated).

upregulation of IL-8R seems to be another such response which stimulates the neutrophils for augmentation of that response.

Colchicine is known to block polymerization of tubulin subunits into microtubule and in many cases it is used as a degranulation blocking agent [20]. The strong inhibitory effect of colchicine on the expression of IL-8 receptor indicates that the granules of PMNs are the possible source of IL-8R during fMLP stimulation.

Since the marker enzyme levels for azurophilic and specific granules were not considerably increased in the supernatant and A23187 could not increase the binding of IL-8 to cells, the possibility of azurophilic, specific and gelatinase granules being the source of IL-8R could be excluded. The sharp rise of alkaline phosphatase level, the marker enzyme of secretory vesicles of PMNs with concomitant increase in IL-8 binding at the same conc of fMLP, indicates that both the enzyme and the receptor may originate from the same compartment of the granules of neutrophil (Fig. 1). It is possible that, upon stimulation with fMLP, the secretory vesicles are mobilised and fused with the plasma membrane. Subsequently, the presynthesized stored receptors of the vesicles expressed on the cell surface and alkaline phosphatase, which is reported to be located on the luminal side of the vesicles is released [21]. As colchicine blocks the mobilization of granules containing the receptor and the enzyme, we obtained reduced IL-8 binding and alkaline phosphatase release when PMNs were treated with colchicine prior to stimulation with fMLP.

Since metabolic blockers were ineffective in blocking the increase in IL-8 binding and colchicine could inhibit both IL-8 binding and enzyme release, it can be suggested that like fMLP, serum free LPS induces expression of IL-8R and alkaline phosphatase release from the secretory vesicle compartments of PMNs. As the LPS induced expression of IL-8R was very low, it seems that serum-free LPS is not a potent stimulant for IL-8R expression from PMNs (Fig. 2).

The induction of receptor expression during SA-LPS stimulation: (a) could not be blocked by colchicine, but was completely blocked by actinomycin D or cycloheximide; (b) the level of alkaline phosphatase in the cultured supernatant was also not increased. This suggests that unlike fMLP and unactivated LPS (serum-free), the source of the induced receptor during treatment of SA-LPS is de novo protein synthesis. Recently, SA-LPS has been shown to induce de novo protein synthesis rapidly [22]. It has been demonstrated that 5–10 min of exposure with SA-LPS was sufficient to release TNFα from monocytes where de novo protein synthesis was the source of TNFα [22]. In our case, during SA-LPS stimulation in PMNs, 30 min seems to be sufficient to trigger induction of IL-8R expression through protein synthesis (Fig. 2).

A lipopolysaccharide binding protein (LBP; mol. wt. 55 kDa) is constitutively present in the plasma or serum which binds LPS to form a LPS-LBP complex [23]. This complex seems to be the active species which induces upregulation of IL-8R. Polymixin-B, an antibiotic which interacts with lipid A moiety of LPS and neutralises the effect of LPS by forming a non pyrogenic complex [24]. This can explain the inability of LPS activated with serum in presence of polymixin-B to induce IL-8R expression.

The surface antigen CD14 is known to be an opsonic receptor for LBP when it is in complex form with LPS [25]. This complex presumably activates the neutrophil through CD14 and induces expression of IL-8R. Addition of anti-CD14 antibody may block the binding site for LBP-LPS complex preventing the induction of IL-8R expression (Fig. 2).

During fMLP and SA-LPS stimulation, new receptor protein appeared on the surface of PMNs which could be detected by anti-IL-8R antibody (Fig. 3). From Scatchard analysis, the receptor numbers in fMLP and SA-LPS stimulated neutrophils

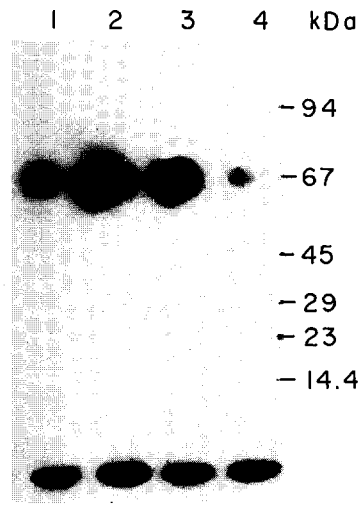

Fig. 5. Autoradiograph of IL-8 receptor chemically crosslinked to [^{125}I]IL-8 from human neutrophils analysed by SDS-PAGE (10%). Lanes 1, 2 and 3 represent control, SA-LPS (10 ng/ml) stimulated and fMLP (10^{-6} M) stimulated neutrophils. Lane 4 indicates that binding of [^{125}I]IL-8 to neutrophils in presence of 50-fold excess cold IL-8. The positions of molecular weight marker proteins are indicated in the figure. The result is representative of three independent experiments.

were found to be considerably higher than in unstimulated cells. But the K_d values for unstimulated and stimulated neutrophils remained within the normal range as reported earlier by us [12].

From the autoradiogram, it was observed that the signal for radioactivity for stimulated cells appeared at 67 kDa. Polyclonal antibody against 58 kDa protein could inhibit 60–70% binding of IL-8 to the cells. Although blocking the receptors with monoclonal antibody against A and B type of receptors can specifically answer this question, from the preliminary study we cannot conclude what type of IL-8R (A or/and B) is expressed by fMLP and SA-LPS. The bands for fMLP and Sa-LPS stimulated PMNs showed more than two-fold increase in intensity with respect to control which was inconsistent with the binding data (Fig. 5). The exact reason for this intense band is not clearly understood. Disappearance of the band in presence of excess unlabelled IL-8 suggests that IL-8 binds to its specific binding protein which was expressed by SA-LPS (Fig. 5).

All the evidence presented indicates that both fMLP and SA-LPS upregulate IL-8R on the surface of PMN. It can be presumed that since neutrophils provide a first line defence against bacterial infection, these bacterial products signal the presence of the invading organisms and stimulate PMNs by rapid alteration of the composition of cell surface proteins. Rapid expression of IL-8R is one such response which may help in the activation of the neutrophils by facilitating the functions of IL-8 for killing the microorganisms at the site of infection from where the fMLP and LPS are generated.

Briefly, we have presented evidence for the first time that the bacterial products fMLP and LPS (serum activated) upregulate IL-8R receptor expression in human neutrophils which may have a potential role in activating the neutrophils during inflammatory response.

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