Mechanism of CD11b down-regulation from phorbol myristate acetate stimulated polymorphonuclear neutrophils

Khalil Abdul-Aziz, *PhD.*

ABSTRACT

Objective: To investigate the mechanism of CD11b down-regulation in phorbol myristate acetate (PMA) stimulated polymorphonuclear leukocytes (PMN).

Methods: Purified PMN were stimulated with PMA in the presence, or absence, of various enzyme inhibitors. Following stimulation, PMN CD11b expression was examined by flow cytometry and Western-blotting. The entire work was carried out at Liverpool University between the period of 1998 and 2001.

Results: Stimulation of PMN with PMA induced the down-regulation of CD11b by a mechanism involving a combination of an oxidant and a serine protease; most likely the primary granule derived elastase.

Conclusion: The present study shows for the first time the cooperation of both oxidants and enzymes in the down-regulation of PMN adhesion receptors.

Saudi Med J 2005; Vol. 26 (10): 1596-1601

Polymorphonuclear leukocytes (PMN, neutrophils) neutrophils constitute the main branch of the innate immune system. In response to inflammatory stimuli, the cells respond by adhering to, and transmigrating across, the endothelium and diapedesis across the extracellular matrix to arrive at the area of inflammation, where they are involved in the phagocytosis and killing of pathogens. These different stages of PMN function are controlled by a number of adhesion receptors interacting with corresponding ligands on the endothelium and the extracellular matrix.[1,2] CD11b/CD18, also known as Mac-1, Mo1 and CR3, is a member of the ß2 integrin family of adhesion receptors that play a major role in PMN physiology. CD11b/CD18 is a heterodimer consisting of an α_m chain (CD11b) and a ß2 chain (CD18) that is shared with other ß2 integrins. The other 2 members of this family are the $\alpha_L/ß_2$ (CD11a/CD18, leukocyte adhesion function-1 [LAF-1]) and the α_x/β_2 [CD11c/CD18, p150,95]).[2,3] In resting PMN, CD11b/CD18 is found stored intracellularly in 3 distinct granules, namely, the secretory vesicles, specific and gelatinase granules.[4,5] Upon PMN activation, the molecule is translocated to the cell surface where it mediates some PMN function including adhesion, migration and phagocytosis.[4,7] In vivo, CD11b/CD18 interacts with the intercellular adhesion molecule-1 (ICAM-1) on the surface of the endothelium, and mediates PMN adhesion to, and transmigration across, the endothelium. In addition, CD11b/CD18 interacts with extracellular components and mediates PMN diapedesis across the interstitium to areas of inflammation, where the molecule interacts with iC3b-opsonised pathogens and facilitates their phagocytosis by PMN.[4] The importance of CD11b/

From the Regional Department of Immunology, Heart of England NHS Foundation, Birmingham, *United Kingdom.*

Received 4th May 2005. Accepted for publication in final form 10th July 2005.

Address correspondence and reprint request to: Dr. Khalil A. Aziz, Senior Clinical Scientist, Regional Department of Immunology, Birmingham Heartlands Hospital, Bordesley Green East, Birmingham B9 5SS, *United Kingdom*. Tel. +44 (121) 4240185. Fax. +44 (121) 4243129. E-mail: khalilazizh@yahoo.co.uk

CD18 in PMN function is clearly evident from patients with leukocyte adhesion deficiency (LAD) syndrome, who suffers from recurrent life threatening bacterial and fungal infections. In these patients, PMN express little or no CD11b/CD18 and, therefore, are unable to adhere to the endothelium and migrate to infected sites.[8] Although, there is a lot of accumulated data on the structure/ function of CD11b/CD18, very little exists on its regulation. We have been interested in studying leukocyte migration across tissues, and the control of such processes.[9-11] While working in this area, we discovered that, following expression on the surface of activated PMN, CD11b/CD18 was subsequently down-regulated through proteolysis, with the resultant loss of its functional activity.[11] The present study, investigates the mechanism of CD11b/CD18 down-regulation and attempt to elucidate the responsible enzyme.

Methods. The following mAbs were used:-anti CD11b, D-12 (IgG2a, Becton Dickinson [BD], Oxford, UK), OKM-1 and 2LPM19c (IgG1; Dakopatts Glostrup, Denmark); anti-CD44-2C5 IgG2a (R&D). Mouse IgG1 and IgG2a were used as isotypic controls. Second layer fluorescent goat anti-mouse Ig (GAM-Ig-FITC; BD) was used. The following reagents were used: phosphate-buffered saline (PBS); Hanks balanced salt solution (HBSS); RPMI 1640 (Life Technologies, Paisley, UK) and monopoly-resolving medium (ICN Biomedicals, Thames, UK). The reagents purchased from Sigma (Poole, UK) were Phorbol 12-Myristate 13-Acetate (PMA); ethylenediaminetetraacetic acid (EDTA); N-methoxy succinyl Ala-Ala-Pro-Val chloromethyl ketone (an elastase inhibitor), phenyl methyl sulphonyl fluoride (PMSF), aprotinin, antipain, chymostatin, leupeptin, pepstatin. Triton X-100 was from BDH (Poole, UK). Reagents for FACS analysis Cell wash; Cellfix and FACScan were obtained from BD. Protein G Sepharose beads (Zymed, San Francisco, CA), immobilon-P membrane (Millipore, Bedford, MA) and enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Amersham, UK). The entire work was carried out at Liverpool University between the period of 1998 and 2001.

Polymorphonuclear leukocytes were isolated from fresh venous blood following dextran-sedimentation of red blood cells and supernatant centrifugation through a gradient of monopoly resolving medium as previously described.[12] The final cell preparation ($1x10^7$ cells/ml in RPMI) contained >98% PMN. For flow cytometry, PMN were fixed in 1% paraformaldehyde in PBS (pH 7.3) immediately following stimulation. Fixed PMN were washed twice with PBS containing 1% bovine serum albumin (BSA), 0.1% azide, pH 7.3 (PBA), then incubated with a primary mAb at saturating concentrations for 30 minutes at 4^0C. Cells were washed twice with PBA prior to incubation with FITC-conjugated anti-mouse IgG for 20 minutes at 4^0C in the dark. Polymorphonuclear leukocytes neutrophils were then washed twice, resuspended in PBA and fluorescence measured using a FACScan (Becton Dickinson).

Polymorphonuclear leukocyte viability before and after stimulation with PMA was determined by trypan blue exclusion and by FACS analysis of cells stained with propidium iodide (PI)[13] (dead cells become permeable to PI and bright red). Cell viability was routinely >98%. For immunoprecipitation stimulated, or unstimulated, PMN suspensions were either lysed with equal volumes of 2x NP 40 lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% nonidet-40 (NP 40), PMSF 4 mM, leupeptin 1 µg/ml, aprotinin 200 units, or centrifuged and the supernatants placed on ice and the following inhibitors added: aprotinin, antipain, chymostatin, leupeptin, pepstatin (all at a final concentration of 5 µg/ml for 60 minutes). These beads were then replaced by fresh washed beads, 5 mg/sample isotypic control antibody added and the mixing continued for another 60 minutes. Supernatants were harvested and incubated at 4^0C overnight with CD11b mAb (5 µg/sample). The Protein G Sepharose beads were then added for one hour at 4^0C. Following incubation at 4^0C for 60 minutes, the cell lysates were centrifuged for 30 minutes at 15,000g at 4^0C. The immune complexes bound to the beads were dissociated by boiling in double strength sample buffer with, or without, 2-mercaptoethanol for 5 minutes prior to electrophoresis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing or non-reducing conditions,[14] using 5-15% gradient gels. Following SDS-PAGE, proteins were transferred to immobilon-P membranes using a Trans-Blot electrophoretic transfer cell (Bio-Rad). Membranes were blocked with 100 mM NaCl, 0.1% Tween-20, 10 mM Tris, pH 7.5 (TBS.T) containing 5% (wt/vol.) nonfat dry milk for 1 hour at 37^0C and then overlaid with 2LPM19c mAb at a concentration of 2 µg/ml in TBS.T containing 1% milk. After overnight shaking incubation at 4^0C, the membrane was washed 3 times with TBS.T and overlaid with horse-radish peroxidase-conjugated antibody at a concentration of 0.2 µg/ml in TBS.T containing 5% milk for 30 minutes at 37^0C. After 3 washes with TBS.T, mAb-labeled proteins were detected using enhanced chemiluminescence reagent.

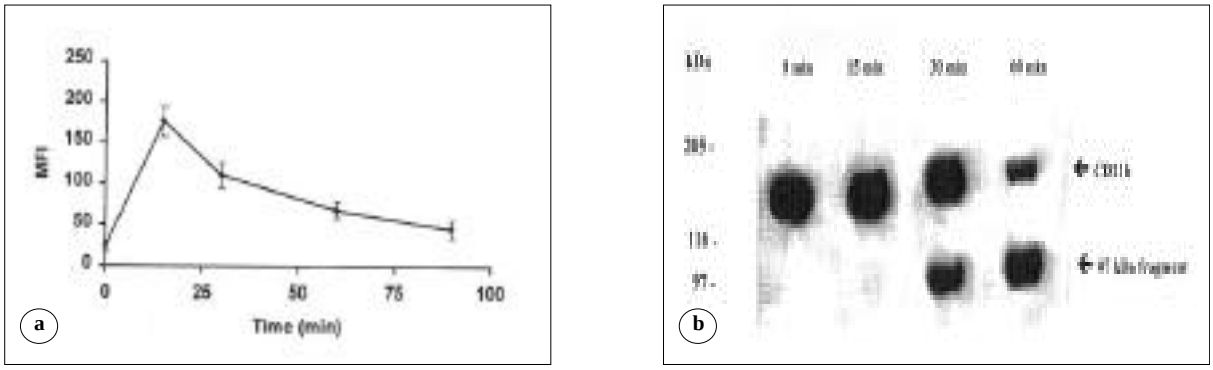

Figure 1 - Time course of CD11b modulation in PMA-stimulated PMN. Polymorphonuclear leukocytes (PMN) were stimulated with PMA (10 ng/ml) at 37°C for various time points. **a)** At indicated points, aliquots of PMN were taken and CD11b expression assessed by the D-12 mAb and flow cytometry. Results are means ± SEM from 3 different experiments. **b)** Aliquots of stimulated PMN were also taken at the indicated points and CD11b assessed by SDS-PAGE and western blotting using the 2LMP19c mAb. Both antibodies are directed against the N-terminal region of CD11b. PMA - Phorbol 12-myristate 13-acetate, MFI - mean fluorescence intensity

Table 1 - Effect of proteinase inhibitors on CD11b down-regulation proteinase inhibitors loss of CD11b.

Proteinase inhibitors	**Loss of CD11b expression***
PMA alone	91 ± 6
PMA+	
PMSF	20 ± 7
EI	87 ± 3
α1-antiprotienase	85 ± 6
Aprotinin	83 ± 11

*CD11b loss is expressed as a % loss; taking the expression at 15 minutes as 100%. Results are means ± SEM from 4 different experiments. PMA - Phorbol 12-myristate 13-acetate, PMSF - phenyl methyl sulphonyl fluoride, EI- elastase inhibitor

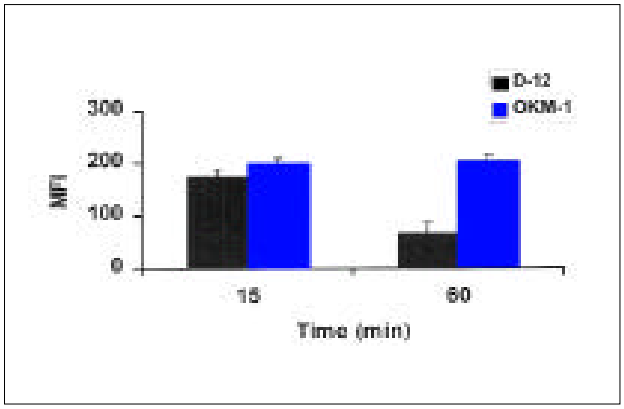

Figure 2 - Effect of Polymorphonuclear leukocytes-stimulation on the binding of different mAbs against CD11b. MFI - mean fluorescence intensity

Results. **Figures 1a & 1b** confirms our previous demonstration that CD11b is down-regulated from PMA-stimulated PMN through proteolysis.[11] **Figure 1a** shows that stimulation of PMN with PMA leads to up-regulation of CD11b/CD18 on the cell surface; peaking at approximately 15 minutes. This is then followed by down-regulation of the molecule to the resting level by 60 minutes from the start of stimulation. Down-regulation of CD11b/CD18 occurred as a result of proteolysis of the distal part of CD11b molecule (**Figures 1b**).[13] Previously, we have demonstrated that the enzyme responsible for CD11b degradation was a serine protease.[13] However, the identity of the enzyme, and the site of degradation, were not determined. The present study attempted to identify the responsible enzyme and the site of degradation. Using a number of enzyme inhibitors, it was observed that cell-permeable serine protease inhibitor (PMSF) greatly reduced the down-regulation of CD11b, but cell-impermeable inhibitors (α1-antiprotinase, aprotinin and elastase-specific inhibitor) had no effect (**Table 1**). These observations strongly suggested that CD11b is degraded in either, an intracellular compartment, or in the cell membrane. To determine the site of degradation, PMN were stimulated with PMA and CD11b expression was then examined on the cell surface using antibodies directed against the proximal region (OKM-1) and the distal cleaved fragment (D-12). **Figure 2** shows that at 15 minutes of incubation, both CD11b epitopes were expressed on the cell surface. In contrast, by 60 minutes of stimulation, the epitope on the distal part of CD11b (D-12) had been lost, but the proximal region

Table 2 - Effect of oxidants in the down-regulation of CD11b.

Inhibitors/scavengers	Loss of CD11b expression*
PMA alone	89 ± 4
PMA +	
Cytochrome C (1 mg/ml)	87 ± 6
Superoxide dismutase (50 µg/ml)	91 ± 5
Catalase (200 µg/ml)	85 ± 3
Azide (1mM)	5 ± 3
Diphenylene iodonium (20 µM)	0
Taurine (2mM)	87 ± 4

Polymorphonuclear leukocytes were incubated alone, or with the above inhibitors/scavengers for 5 min at 37°C before stimulation with Phorbol 12-myristate 13-acetate (PMA) and CD11b measurements. Results are means ± SEM from 4 different experiments

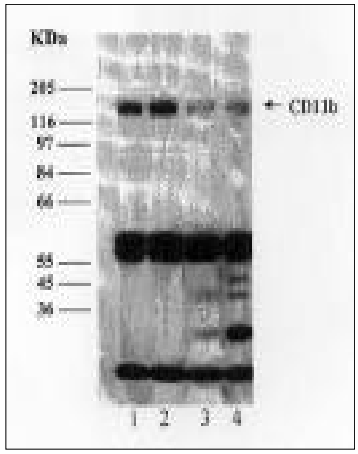

Figure 4 - Effect of candidate enzymes on immunoprecipitated CD11b. Lane 1 - immunoprecipitated CD11b was incubated at 37°C for 60 minutes with RPMI, Lane 2 - supernatants from unstimulated polymorphonuclear leukocytes (PMN), Lane 3 - with supernatants from Phorbol 12-myristate 13-acetate-stimulated PMN and Lane 4 -with purified elastase (0.02 units/ml).

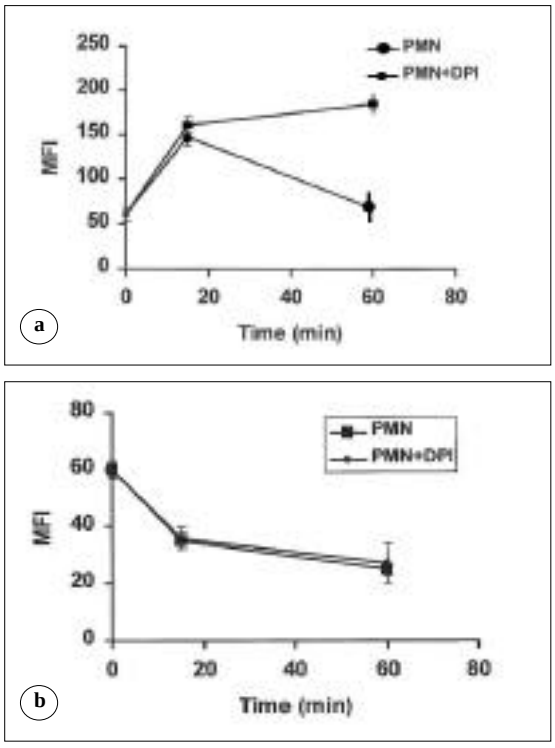

Figure 3 - Effect of oxidants on PMN receptor expression. Aliquots of PMN were removed and stained for **a)** CD11b with the D-12 mAb or **b)** CD44 with the 2C2 mAb. PMN - polymorphonuclear leukocytes, DPI - diphenylene iodonium, PMA - Phorbol 12-myristate 13-acetate, MFI - mean fluorescence intensity.

(OKM-1) was still fully expressed. It was noted that a delay of approximately 20 minutes occurred between the start of PMN stimulation and the start of CD11b degradation. The start of degradation followed the peak of oxidant production (results not shown). These observations suggested that oxidants, aside from a serine protease(s), could play a role in the degradation of CD11b. Therefore, the role of oxidants in CD11b degradation was examined. **Table 2** shows that a cell-permeable specific inhibitor of PMN NADPH-oxidase, diphenylene iodonium (DPI), completely inhibited the down-regulation of CD11b. Inclusion of a cell permeable myeloperoxidase (MPO)-specific inhibitor, azide, also completely inhibited the down-regulation of CD11b. However, scavenging extracellular super-oxide anion with Cytochrome C, or destruction of extracellular superoxide and hydrogen peroxide by addition of superoxide dismutase (SOD) and catalase; had no effect on CD11b down-regulation. Moreover, neither removal of secreted MPO, nor scavenging of extracellular MPO-product (hypo-chlorous acid) with taurine, had any effect on CD11b down regulation (**Table 2**). In contrast to CD11b down-regulation, DPI and azide had no effect on PMA-induced down-regulation of CD44 (**Figures 3a & 3b**) and CD43 (results not shown). **Figure 4**, lane 3, shows that enzymes secreted from PMA-stimulated PMN caused substantial degradation of purified CD11b bound to immunobeads. This effect could be inhibited by elastase specific inhibitor (results not shown) and reproduced with purified elastase (**Figure 4, lane 4**).

In contrast to elastase, Cathepsin G had no effect on purified CD11b degradation (results not shown).

Discussion. The present study investigates the mechanism of CD11b/CD18 down-regulation from stimulated PMN. The study shows that, following expression on the cell surface, CD11b is down-regulated by a mechanism involving proteolytic degradation. Polymorphonuclear leukocytes contain some serine proteases stored in their primary granules, prominent amongst these are cathepsin G and elastase. Polymorphonuclear leukocytes stimulation release these enzymes from the primary granules into intracellular compartment (phagosomes), incorporated into the cell membrane, or released extracellular. Since we, and others, have previously shown that secreted, and purified, elastase degrades a wide variety of different proteins,[15,16] this enzyme seemed a likely candidate in the degradation of CD11b. However, inhibition of secreted elastase (and cathepsin G) had no effect on CD11b down-regulation. Moreover, addition of purified elastase (and cathepsin G) to mildly stimulated PMN did not induce CD11b down-regulation. These results excluded the role of secreted enzymes (including elastase and cathepsin G) in the down-regulation of CD11b, and pointed towards the role of cell associated enzyme(s). The use of cell permeable, and impermeable, enzyme inhibitors further strengthened the role of cell associated serine protease(s) in the degradation of CD11b. Since elastase can become incorporated into the cell membrane the above results, although ruled out the involvement of secreted elastase, did not rule out the involvement of cell associated elastase- and cathepsin G- enzymes. To investigate the precise site of CD11b degradation, we used 2 monoclonal antibodies; one directed against the distal cleaved region of CD11b, and another against the undegraded proximal part of the molecule. Internalization of CD11b into intracellular compartment would be expected to result in the down-regulation of the whole molecule, whereas degradation of CD11b on the cell surface would only result in the down-regulation of the distal cleaved part. In a time course study of CD11b, the distal part of CD11b was down-regulated, whereas the proximal uncleaved part of the molecule remained constant through out the period of stimulation (**Figure 3**), indicating that proteolysis of CD11b occurred on the cell surface, instead of in intracellular compartment. Incorporation of both elastase and cathepsin G into PMN surface membrane has been previously demonstrated. These enzymes would be closely associated with CD11b molecule and less likely to be inhibited by extracellular enzyme inhibitors and, therefore, could play a role in the proteolysis of CD11b. We observed that there was a delay between the start of PMN-stimulation and CD11b down-regulation. Moreover, we also observed that the start of CD11b down-regulation coincided with maximum PMN oxidant production. These observations strongly suggested the involvement of oxidants, in addition to serine protease(s), in the down-regulation of CD11b. Stimulation of PMN leads to activation of the cell membrane associated NADPH-oxidase, which reduces molecular oxygen to super-oxide anion, a highly unstable oxidant radical that spontaneously dismutates to hydrogen peroxide. The primary granule derived myeloperoxidase (MPO) then utilized the latter product to produce hypochlorous acid, a highly active radical capable of inducing structural changes in many proteins, enzymes and lipids.[17,18] Inhibition of the NADPH-oxidase- and the MPO-enzymes with cell permeable DPI and azide, prevented the down-regulation of CD11b. However, scavenging extracellular superoxide anion, hydrogen peroxide and hypochlorous acid had no effect on CD11b down-regulation. These results implicated the NADPH-oxidase-MPO derived hypochlorous acid in the down-regulation of CD11b. Prevention of CD11b down-regulation by both DPI and azide can not be attributed to toxic effects on PMN, since both agents had no effect on other PMN function including degranulation and chemotaxis.[19,20] In addition, both DPI and azid had no effect on the down-regulation of other PMN surface receptors including CD44 and CD45. Hypochlorous acid is well known to induce structural changes in a wide variety of serum proteins and enzymes, thereby, making them susceptible to subsequent degradation by proteolytic enzymes.[21,24] The present study indicates that hypochlorous acid induces conformational changes in the structure of CD11b molecule thereby making it susceptible to subsequent degradation by a cell associated serine protease(s). Alternatively, hypochlorous acid could induce CD11b down-regulation indirectly through activation of cell associated serine proteinase. Since it was not possible to identify the serine enzyme responsible for CD11b degradation in intact cells, CD11b was purified and the effect of various PMN enzymes on the molecule studied. We observed that secreted enzymes, although had no effect on CD11b in intact PMN, resulted in substantial degradation of purified CD11b. The effect of secreted enzymes on CD11b could be inhibited by elastase specific inhibitor and reproduced with purified elastase. These observations strongly suggested that binding of CD11b to Immunobeads induced conformational changes in the molecule which became susceptible to subsequent degradation by elastase. Taken together, these results indicate that changes in CD11b structure, whether induced by exposure to oxidants, or through binding to immunobeads, are needed before the molecule becomes susceptible to degradation by elastase. The precise site of CD11b degradation remains to be elucidated.

In conclusion, the present study shows for the first time that CD11b is proteolytically down-regulated from stimulated PMN by a mechanism involving oxidant and a serine protease; most likely the primary granule derived elastase.

References

1. Cramer EB. Cell biology of phagocyte migration from bone marrow, out of the blood stream, and across epithelium. In: Gallin JI, Goldstein IM, Snyderman R, editors. Inflammation: Basic Principals and Clinical Correlates. New York: Raven Press; 1992. p. 341-351.
2. Arnaout MA. Structure and function of leukocyte adhesion molecules CD11b/CD18. ***Blood*** 1990; 75: 1037-1050.
3. Carlos TM, Harlen JM. Leukocyte-endothelial adhesion molecules. ***Blood*** 1994; 84: 2068-2101.
4. Arnaout MA, Todd RF, Dana N, Melamed J, Schlossman SF et al. Inhibition of phagocytosis of complement C3- or Immunoglobulin G-coated particles and of iC3b binding by monoclonal antibodies to monocyte-granulocyte membrane glycoprotein (Mo1). ***J Clin Invest*** 1983; 72: 171-176.
5. Etzioin A. Adhesion molecules their role in health and disease. ***Paediatric Research*** 1996; 39: 191-198.
6. Kjeldsen L, Sengelov H, Lollike K, Nielsen MH, Borregaad N. Isolation and characterisation of gelatinase granules from human neutrophils. ***Blood*** 1994; 83: 1640-1649.
7. Sengelov H, Kjeldsen L, Diamond MS, Springer TA, Borregaard N. Subcellular localisation and dynamics of mac-1 (αmß2) in human neutrophils. ***J Clin Invest*** 1993; 92: 1467-1476.
8. Springer TA, Thompson WS, Miller LJ, Schmalstieg FC, Anderson DC. Inherited deficiency of the Mac 1, LFA-1, p150, 95 glycoprotein and its molecular basis. ***J Exp Med*** 1984; 160: 1901-1918.
9. Aziz KA, Till KJ, Zuzel M, Cawley JC. Involvement of CD44-hyaluronan interaction in malignant cell homing and fibronectin synthesis in hairy cell leukemia. ***Blood*** 2000; 96: 3161-3167.
10. Aziz KA, Till KJ, Chen H, Slupsky JR, Campbell F, Cawley JC et al. The role of autocrine FGF-2 in the distinctive bone marrow fibrosis of hairy-cell leukaemia (HCL). ***Blood*** 2003; 102: 1051-1056.
11. Davey PC, Zuzel M, Kamaguti AS, Hunt JA, Aziz KA. Activation-dependent proteolytic degradation of polymorphonuclear CD11b. ***Br J Haematol*** 2000; 111: 934-942.
12. Aziz KA, Cawley JC, Zuzel M. Platelets prime PMN via released PF4: mechanism of priming and synergy with GM-CSF. ***Br J Haematol*** 1995; 91: 846-853.
13. Migliorati NG, Pagliacc, MC, Grignani F, Riccardd C. A rapid and a simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. ***J Immunol Methods*** 1992; 139: 271-279.
14. Laemli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. ***Nature*** 1970; 227: 680-685.
15. Aziz KA, Cawley JC, Zuzel M. Degradation of platelet GPIb by elastase released from primed neutrophils. ***Br J Haematol*** 1994; 91: 46-54.
16. Remold-O'Donnell E, Parent D. Specific sensitivity of CD43 to Neutrophil elastase. Blood 1995; 86: 2395-2401.
17. Babior BM. NADPH Oxidase: An update. ***Blood*** 1999; 93: 1464-1476.
18. DeChatelet LR, Long GD, Shirley PS, Bass DA, Thomas MJ, Henderson FW et al. Mechanism of the luminol-dependent chemiluminescence of human neutrophils. ***J Immunol*** 1982; 129: 1589-1593.
19. Cross AR, Jones TG. The effect of the inhibitor diphenylene iodium on the superoxide-generating system of neutrophils. ***J Biochem*** 1986; 237: 111-116.
20. Cross AR. The inhibitory effects of some iodium compounds on the superoxide generating system of neutrophils and their failure to inhibit diaphorase activity. ***Biochem Pharmacol*** 1987; 36: 489-493.
21. Discipio RG, Daffern PJ, Kawahara M, Pike R, Travis J, Hugali TE et al. Cleavage of human complement C5 by cysteine proteinase from Porphyromonas (bacteroides) ginfivalis. Prior oxidation of C5 augments proteinase digestion of C5. ***Immunol*** 1996; 87: 660-667.
22. Hawkins CL, Davies MJ. Hypochlorite-induced damage to proteins: formation of nitrogen-centred radicals from lysine residues and their role in protein fragmentation. ***J Biochem*** 1998; 332: 617-625.
23. Reddy VY, Desorchers PE, Pizzo SV, Gonias SL, Sahkian JA, Levine RL et al. Oxidative dissociation of human alpha 2-macroglobulin tetramers into dysfunctional dimers. ***J Biol Chem*** 1994; 269: 4683-4691.
24. Varani J, Ginsburg I, Schuger L, Gibbs DF, Bromberg J, Johnson KJ, et al. Endothelial cell killing by neutrophils. Synergistic interaction of oxygen products and proteases. ***Am J Pathol*** 1989; 135: 435-438.