Impaired cell motility in chronic myeloid leukemic granulocytes related to altered cytoskeletal pattern

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The bactericidal activity of polymorphonuclear leucocyte (PMNL) against infection stimulates cytoskeletal changes accompanied with alteration in adhesion and locomotion. Microfilaments, the motile apparatus is known to regulate these changes by polymerization of monomeric G-actin to fibrous F-actin. PMNL from chronic myeloid leukemia (CML) patients have been reported to be defective in locomotion in response to synthetic peptide, n-formyl-methionyl-leucyl-phenylalanine (fMLP) but the mechanism leading to defective locomotion and their spatial reorganization remains unclear. Therefore, in order to study the cause of defective motility of PMNL from CML patients the spatial distribution and reorganization of microfilaments and microtubules in response to fMLP have been examined by transmission electron (TEM) and scanning electron microscopy (SEM). Under SEM, the PMNL-CML surface appeared smoother with reduced ruffling resulting in rounding off cells with lesser polarized morphology. Unstimulated PMNL from normal as well as CML subjects showed shorter and fewer microtubules and evenly distributed microfilaments as compared to fMLP stimulated PMNL. It is proposed that the cause of defective locomotion was due to reduced surface activity as a consequence of altered cytoskeletal configuration. This phenomenon seems to be related to impaired functional appendages and as a whole led to the defective cell motility and hence reduced chemotaxis in PMNL from CML patients.

Keywords: Bactericidal activity, Cell motility, Chronic myeloid leukemia, Cytoskeletal pattern, PMNL

The human polymorphonuclear leucocytes (PMNL) play an essential role in host defence mechanism against pathogenic micro-organisms. Fulfilment of this role requires a co-ordinated series of motile functions such as diapedesis, chemotaxis, secretion and phagocytosis. The major events involved in microbicidal activity are sensing of a source of organisms mediated through the actions of chemoattractants followed by orientation of cells and their directional movement leading to phagocytosis. In eukaryotic cells, microfilaments (MF) and microtubular (MT) assembly is known to play a major role in initiation of cell motility. Therefore, PMNL is an ideal cell system to study motility in response to different chemotactic factors such as n-formyl-methionyl-leucyl-phenylalanine (fMLP), C5a, and leukotrine B4 (Ref. 4). The lamellipodium and uropod are the best sites of rapid polymerization and re-organization of actins. MFs act as a motile apparatus and microtubules on the other hand are required to maintain neoformation of these appendages through their co-ordination and/or assembly. The motile behaviour of PMNL from normal and chronic myeloid leukemia (CML) subjects in response to fMLP has been documented. Increased thickness of microtubules in PMNL from CML after stimulation with fMLP has been reported to reduce adhesiveness, motility and decreased actin polymerization. Low availability of chemotactic receptor and even lower amount of actin may influence the fMLP induced thickness of PMNL from CML patients. These studies suggested that both elongation of pre-existing and nucleation of microtubules may contribute to fMLP induced motility. It is also well known that microtubules tend to polymerize from microtubule organizing centre (MTOC) located anterior to the nuclear lobe extending toward the periphery of cell. Topographical re-organization of plasma membrane is associated with cytoskeleton where MFs and MTs play a major role. However, lack of topographical evidence regulating the fate of MFs and MTs in both normal and PMNL CML is not yet clear. This led us to
investigate the surface changes occurring in PMNL CML by SEM study using fMLP as a chemoattractant. In addition, an attempt has been made to correlate the alteration or defects in MFs and MTs by conventional as well as special transmission electron microscopy methods.

**Materials and Methods**

**Chemicals**—n-Formyl-methionyl-leucyl-phenylalanine (fMLP), sodium di-trizoate (hypaque), poly-L-lysine, osmium tetroxide, trypsin and benzoyl-dL-arginine-p-nitroanilide [BAPNA] were obtained from Sigma Chemical Co. USA. Ficoll was purchased from Pharmacia Fine Chemicals, Uppsala Sweden.

**Preparation of human neutrophils (PMNL)**—Blood from healthy subjects and CML patients, 48 each, was collected simultaneously for preparation of PMNL before the commencement of therapy. CML patients were diagnosed on standard clinical-haematological parameters at the Tata Memorial Hospital (Mumbai). PMNL were isolated on a ficoll-hypaque gradient according to the method of Boyum. Samples with cell viability above 90% as seen by dye exclusion with 0.4% erythrocin-B were allowed to adhere on poly-l-lysine coated plastic petridishes for 20 min at 37°C. Cell suspension containing 1X 10^7 cells/ml from both healthy and CML patients were added to each petridish. Same number of cells were stimulated with fMLP at a concentration of 10^{-8}M in DMSO for 20 min at 37°C and unstimulated cells served as DMSO control. Orientation of cells was observed under phase contrast microscope. Samples were fixed in 2% glutaraldehyde and osmium tetroxide for 45 min at 4°C and processed for electron microscopy in situ according to Brinkley. Flat embedded cells were sectioned either parallel or perpendicular to their substratum. Serial sections were picked up on formvar coated grids, stained with uranyl acetate and lead citrate, sections on grids were observed under the Zeiss 109, Electron Microscope at 80Kv.

**Myosin extraction**—Myosin was purified from rabbit dorsal leg muscles according to Szent Gyorgy’s method in ice cold milli-Q water with 3 cycles of crystallization. The purity of myosin was checked by SDS-PAGE. HMM was isolated from myosin with digestion of trypsin, the concentration of which was determined by using BAPNA as a substrate according to standard methods. HMM immediately after separation was used in vitro decoration of filaments in PMNL by the method of Goldman. Briefly, the PMNL after washing with standard salt solution at pH 7 were permeabilized with 5% glycerol for 60 min at 22°C in 0.07 M potassium phosphate buffer pH 7 containing 1-2 mg/ml Heavy meromyosin (HMM). The controls for HMM decoration for the above cells were simultaneously carried out without adding HMM in the final step. All samples were washed with 0.05M KCl, and fixed in mixture of glutaraldehyde and osmium tetroxide and further processed for electron microscopy.

**Immunogold electron microscopy**—Microtubules (MTs) were visualized according to the standard methods. In short, the PMNL from normal individuals as well as from CML patients after adherence to poly-l-lysine coated dishes were stimulated by fMLP (10^{-8}M) and controls with DMSO alone for 25 min at 37°C. These PMNL were followed by permeabilization with 0.1% triton-X 100 in PBS for 1 min. These permeabilized cells were fixed in both freshly prepared formaldehyde and glutaraldehyde for 20 min. After washing with PBS, the cells were incubated at 37°C for 1 hr with monoclonal antibody to tubulin (diluted 1:200) followed by the secondary antibody labelled with protein A gold for 1 hr at 37°C. The above cells were fixed in glutaraldehyde and postfixed in osmium tetroxide for 45 min and processed for transmission electron microscopy as described earlier.

**Scanning electron microscopy (SEM)**—Cell suspension (50μl) containing 1×10^7 cells/ml was spread on previously poly-L-lysine coated coverslips. Coverslips were kept in moist chamber containing PBS and subsequently fixed in 2% glutaraldehyde at 4°C. After postfixation in 1% osmium tetroxide the coverslips were dehydrated through graded ethyl alcohol and dried in liquid CO₂ at critical point in a polaron criticalc point drying apparatus (CPD) at specific pressure and temperature (31.2°C) as described by Anderson. Sputter coating of coverslips was done with gold palladium in polaron E-5000 sputter coater as described by Kessel and cells were observed by JSM-25S II, JEOL scanning microscope at 5 kv.

**Results**

**Phase contrast microscopy**—As shown by phase contrast microscopy, PMNL from healthy subjects stimulated with fMLP showed more polarized and oriented morphology (Fig. 2B) as compared to unstimulated PMNL (Fig. 2A). The unstimulated
PMNL CML showed rounding off the cells, loss of spreading and maintaining adherence to some extent to substratum (Fig. 2C). These PMNL CML when stimulated with fMLP acquired polarized and oriented morphology (Fig. 2D). The polarization and orientation of PMNL stimulated with fMLP was found to be significantly higher than that of PMNL CML as shown in scattergram (Fig.1). The scattergram thus depicted was just to show the approximate percentage of oriented cells and not for statistical significance.

Scanning electron microscopy—Scanning electron microscopy revealed round PMNL with irregular shape possessing a number of membrane ruffles, small pseudopods and elongated tails (Fig. 2E). In contrast, PMNL from CML patient appeared more smoother and rounded except few rudimentary ruffles (Fig. 2F). PMNL stimulated with fMLP on higher magnification displayed extended lamellipod with increased number of elevations of membrane with full of ruffles and ridges (Fig.3A). PMNL CML even after fMLP stimulation induced their orientation to some extent with smoother lamellipod bearing fewer ruffles and ridges (Fig.3B). The SEM observations were represented in scattergram (Fig.1) which depicted that stimulated oriented PMNL were four fold higher than the stimulated PMNL from CML patients.

General transmission electron microscopy—Generally 12 to 15 cells per section were counted from each grid at low magnification for differential counting of rounded and oriented cells. TEM revealed a number of elongated appendages extending from surface of both PMNL stimulated and unstimulated PMNL. But pattern of distribution of microfilaments in both was different. The stimulated PMNL showed spatial re-organization and polymerization of MFs (Fig. 3F), whereas the unstimulated PMNL showed monomeric actin like microfilaments evenly distributed within the domain of pseudopods (Fig.3C). On the contrary, PMNL CML observed by TEM showed stubby and short attachment points with homogenous distribution of monomeric actin like microfilaments in the form of aggregates (Fig.3D). These PMNL CML after stimulation with fMLP induced moderately elongated appendages but failed to re-organization and/or polymerization of MFs (Fig.3F) as compared to Fig. 3E.

Heavy mero myosin (HMM) decoration studies—Actin filaments as seen by conventional TEM were confirmed by HMM decoration to determine the direction of movement as per the standard methods26,27. With HMM decoration aggregates of filaments were confined at the periphery of unstimulated PMNL, whereas PMNL CML had hardly detectable HMM decoration at peripheral region. In HMM negative control i.e. without HMM in the final step, the PMNL from healthy subjects did show few filaments which were absent in PMNL CML patients (unpublished observation). After stimulation with fMLP, PMNL when treated with HMM showed fuzzy appearance (decoration) of actin filaments were found to be associated with lamellipod, uropod, attachment points and ruffles (Fig.4A) in healthy subjects. In contrast, PMNL from CML patients showed moderate HMM decoration of actin filaments in these regions (Fig.4B).

Immunogold electron microscopy—MTs as stained with tubulin specific antibody revealed short and few MTs in PMNL originating from MTOC (Fig.4C), whereas PMNL from CML showed fewer MTs (Fig.4D). Upon stimulation with fMLP, the PMNL showed straight, longer MTs (Fig.4E) while fine network of collapsed MTs was observed in PMNL CML (Fig.4F). Overall, the number of microtubules found in fMLP treated PMNL and PMNL CML were increased as compared to their unstimulated counterparts.

Discussion

The PMNL are unique regarding their different type of defensive functions. The mechanisms related to the defective or impaired functions of PMNL in various diseases including CML are not yet very clear. This is because the stimulatory factors vary in
Fig. 2—Phase contrast micrograph of (A): PMNL from normal healthy subjects. [PMNL showing even spreading and rounding on substratum, a typical feature of resting cells (× 600)]. (B): PMNL from normal subjects stimulated with fMLP. Majority of cells show polarized cell morphology (× 600). (C): Unstimulated PMNL from CML patients. [PMNL showing less spreading and adherence but retained rounded morphology (× 600)] and (D): fMLP stimulated PMNL from CML patients. [PMNL showing less oriented morphology but more of spreading (× 600)]. (E): SEM of unstimulated PMNL from normal healthy subjects showing evenly distributed ruffles (R) all over the surface [Also note a small pseudopod (p) with extended tail (T) (× 8000)] and (F): SEM of unstimulated PMNL from CML patient showing reduced number of ruffles (R) and smoothening of surface (× 8000).
Fig. 3 (A)—SEM at higher magnification showing distinct broad lamellipod with a number of elevations (E) of membrane in fMLP stimulated normal subjects (× 14800). (B): Orientation of PMNL from CML patients after fMLP stimulation [Note reduction in size of lamellipod, reduced elevations (E) and absence of ruffles is clearly seen (× 14000)]. TEM of (C): unstimulated PMNL cell from healthy subjects, PMNL showing more number of extended appendages as attachment points (× 5000). (D): Unstimulated PMNL from CML patient showing retraction of membrane with reduced number of attachment points as compared with fig 3C [Note homogenous distribution of actin at periphery of the cell (× 5000)]. (E): TEM of fMLP stimulated PMNL from healthy subjects [Aggregates of actin filament (→) at lamellipod region, as shown in the Fig. 2A (by SEM). Note no granules seen at periphery (× 40000)]. (F): TEM of fMLP stimulated PMNL from CML patient. [A representative lamellipod showing absence of aggregate of actin filament as shown in contrast to Fig. 3E with few granules at periphery (× 40000)].
Fig. 4—TEM of (A) HMM decoration in PMNL from healthy subjects stimulated with FMLP [Fuzzy appearance of MFs network is seen as arrowhead structures (→) due to HMM binding (× 75000)]. (B): PMNL from CML patient stimulated with FMLP showing moderately decorated actin filaments (→). (× 75000). (C): Immunogold electron micrograph of unstimulated PMNL from healthy subjects showing short and few MTs (→) originating from MTOC (× 75000). (D): Unstimulated PMNL from CML patient showing shorter and fewer MTs (→) originating from MTOC. (× 38000). (E): FMLP-stimulated PMNL from healthy Subjects [An increased number of distinct, straight and elongated MTs (→) extending to cell periphery are clearly visible. (× 40000)] and (F): PMNL from CML patients stimulated with FMLP. [A fine network of decreased MTs(→) is seen as diffused and collapsed MTs assembly (×38000)].
their capacity to elicit different leukocyte functions. However, factors stimulating or blocking their motility have been reported. These factors stimulate cellular adhesion to substratum chemotaxis, phagocytosis, oxidative burst as well as inducible type of changes in cellular shape. The visible changes in their shape in response to different stimuli have been investigated. Three dimensional changes in PMNL shape have been investigated by laser confocal microscopy in response to chemotactic factors such as fMLP.

Microfilament and microtubules are known to play a major role in orderly regulating their reorganization during changes in cellular shapes. In the present study, more polarized and oriented morphology has been shown in PMNL than PMNL CML induced with fMLP which is in good agreement with others. There is an increased adherence and motility stimulated with fMLP in PMNL CML compared to its control indicating that fMLP may transiently induce small appendages as attachment points. Overall, defective PMNL from CML patient retain capacity to adhere to some extent since all of these events may be concurrently occurring in cells responding to fMLP which induce impaired motility.

A number of mechanisms have been proposed for defective chemotaxis which include formation of filopodia with reorganization of actin containing filaments approaching towards the periphery. Pederson suggested that the impaired phagocytosis and bactericidal capacity are due to the prolonged circulation of incompletely mature neutrophils. Alternatively, it is possible that cells although intrinsically normal (as shown by increased adhesiveness) and their phenotypic abnormalities could be due to alteration in the physiological environments which are secondary to the leukemic crisis. Secondly, it is also well established that subpopulations of PMNL with or without receptors are effective in phagocytosis, bacterial killing, chemotaxis, aggregation and heterogeneous nature of normal and pathogenic subpopulation of circulating neutrophils in CML. These observations suggest that defective polymerization and structural collapse of MTs failed to induce appendages through the MFs required to maintain their spatial architecture. Individual MTs were visualized by immunofluorescence microscopy and fMLP treatment which induced elongation of pre-existing MTs present in unstimulated PMNL by polymerization.

In the present study, the fMLP stimulated PMNL showed thick bundles of MTs, whereas the PMNL CML after fMLP induced MTs, not in the form of...
bundles, but in the form of fine network. These findings indicated that MTs in the PMNL from CML are inducible as straight and elongated as compared to PMNL alone leading to defective cellular shape and motility. There are several reports on types of MTs in PMNL from normal subjects and in different pathological conditions. There are variations in MTs counts and intensity brightness depending on technique used\textsuperscript{11,29,43-45}. Abnormal MT regulation appears to be correlated to abnormal cyclic nucleotide metabolism in Chediak-Higashi syndrome\textsuperscript{44} with depletion of glutathione and decreased MT assembly\textsuperscript{43}. The MTs in human neonates under pathological conditions were diminished with decreased level of cAMP affecting the locomotion\textsuperscript{46}.

In the present study, the number of MTs were found to be shorter and significantly reduced in PMNL from CML patients compared to healthy subjects (Fig. 4D). These short MTs failed to reach to the periphery and may not be able to maintain polarized form during locomotion. This may lead to less polarized cells in fMLP stimulated PMNL from CML patients as well as less perturbation and/or protrusions at cell surface seen by SEM. Based on these observations a more clearer classification of different types of MTs and their thickness using SEM and special TEM will give us to reveal not only their number but also their spatial configuration.

Holt and Koffer\textsuperscript{47} in their review pointed out the importance of proteins rich in proline localized preferably in protrusions. Further, profilin the actin binding protein is known to promote growth and polymerization of actin which also utilize poly-l-proline\textsuperscript{48}. Therefore, the possibility of regulation of cell motility and defective surface activity in PMNL CML by profilin cannot be excluded. Vasodilator stimulated phosphoprotein (VASP) a well established ligand to profilin is known to induce focal adhesions by nucleation of actin\textsuperscript{49} and such ligand binding to actin protein may play a role in regulation of actin polymerization and reformation of attachment points. Reduced surface ruffles and smooth PMNL CML (Fig.3B) even after fMLP stimulation indicates reduced adhesion as a consequence of defective actin nucleation which may be due to lowered binding VASP like ligand to actin binding protein such as profilin. These ruffles found by SEM in the present study suggest their role in endocytosis depending on actin polymerization. Borisey and Svitkingna\textsuperscript{50} pointed out that actin polymerization provides driving force for such protrusions including membrane ruffles. Further, the reduced ruffles and thinning of actin cortex not only reduced adherence but also endocytosis, may be due to defective spatial organization of both MFs and MTs. Similarly, Damiani et al.\textsuperscript{51} reported thinning of such actin cortex by using inhibitors of F-actin which resulted in decreased phagocytosis due to depolymerization of MFs and MTs. These studies suggested that protrusions are essential for cell motility which are regulated by monomeric and polymeric actins. However, in the present study, the MTs that never reached the periphery of PMNL from CML patients could not induce the protrusions. Secondly, the moderately accumulated monomeric or short MFs failed to enhance the motility. Therefore, improper translocation of MTs and polymerized MFs in PMNL from CML patients are correlated to their role in formation of protrusions in former and motility in latter as evidenced by classical EM studies. Deficiency as well as the nature of peptide responsible for motility yet remains unclear.

In summary, the present results indicate first that proper rearrangement of MFs beneath the plasma membrane is required for induction of lamellipodia responsible for cell motility. Secondly, impaired MFs and MTs assembly failed to maintain cellular shape and their appendages because of poorly re-organized and collapsed MTs in PMNL CML. Such type of studies at molecular level were evidenced by reduced adherence of PMNL due to lack of E-selectin, despite of ICAM-1 expression and IL-8 production\textsuperscript{52,53}. Finally, mere presence of MFs and MTs may not be determinants of the adhesion and cell shape, but their spatial configuration, factors responsible for their polymerization have to be ruled out. Taken together, PMNL of CML patients at molecular level will give better insight into the mechanism of defective motility when laser confocal microscopical results are correlated with SEM and TEM findings.

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