Chemotaxis of Blood Neutrophils from Patients with Primary Ciliary Dyskinesia

Primary ciliary dyskinesia is characterized by chronic upper and lower respiratory infections which are caused by the grossly impaired ciliary transport. Since the cilia and neutrophils both utilize microtubular system for their movement, it has been speculated that neutrophil motility such as chemotaxis might be impaired in patients with primary ciliary dyskinesia. Neutrophils were purified from whole blood from 16 patients with primary ciliary dyskinesia and from 15 healthy controls. Chemotactic responses of neutrophils to leukotriene B4 (LTB4), complement 5a (C5a), and formylmethionylleucylphenylalanine (fMLP) were examined using the under agarose method. The chemotactic differentials in response to LTB4, C5a, and fMLP in neutrophils from the patient group were significantly lower than the corresponding values in neutrophils from the control group (p<0.05 for all comparisons). The difference in chemotactic index between the two groups was statistically significant for LTB4 and fMLP (p<0.05 for both comparisons), but not for C5a (p=0.20). Neutrophils from patients with primary ciliary dyskinesia showed a decreased chemotactic response as compared with those from normal subjects. It is concluded that the increased frequency of respiratory tract infection in patients with primary ciliary dyskinesia is possibly due to the defective directional migration of neutrophils, as well as to the defective mucociliary clearance of the airways.

Key Words: Ciliary Motility Disorders; Neutrophils; Chemotaxis

INTRODUCTION

Primary ciliary dyskinesia, formerly referred to as immotile cilia syndrome, is an autosomal recessive disorder. It is characterized by chronic upper and lower respiratory tract infections, which are caused by the grossly impaired mucociliary transport (1). This functional defect results from abnormalities in microtubule structure and arrangement in cilia (2, 3).

Neutrophils are highly motile cells and the most frequent immigrant cells in inflammatory lesions. Their motility requires interaction of contractile proteins in the cortical cytoplasm such as actin, myosin, and actin-binding proteins (4), but morphological as well as pharmacological evidence also points to an important role of microtubule in the regulation of their motility (5, 6). Since the cilia and neutrophils both utilize the microtubular system for their movement, several studies have investigated neutrophil migration in patients with primary ciliary dyskinesia (7-9). However, conclusions drawn from these studies have been variable.

Chemo taxis, the directional locomotion of cells towards a source of a chemical gradient, has been accepted as an important mechanism of mobilizing phagocytes such as neutrophils at sites of infection, tissue injury, and immune reaction (10).

Thus the chemotactic response of neutrophils may be used as a model for cell migration. In this report, we describe the chemotactic response of neutrophils purified from patients with primary ciliary dyskinesia toward optimal concentrations of the following chemotactic agents for the neutrophil: leukotriene B4 (LTB4), Complement 5a (C5a), and formylmethionyllleucylphenylalanine (fMLP).

PATIENTS AND METHODS

We studied 16 children with primary ciliary dyskinesia (9 boys; median age, 11 yr; range, 7 to 16 yr), of whom 4 had Kartagener's syndrome and 7 comprised three groups of siblings (Table 1). The diagnosis of primary ciliary dyskinesia was suspected on clinical grounds and proved by the appearance of cilia on electron microscopy. In all patients, respiratory symptoms had started in early childhood, and they all presented with typical symptoms of bronchitis, recurrent sinusitis or otitis. Bronchiectasis was documented by high-resolution computed tomography in 10 patients. Four patients exhibited clubbing. The cilia were taken from a mucosal biopsy of the inferior nasal turbinate in the absence of local infection. Their
Table 1. Summary of clinical data of 16 patients classified according to the type of primary ciliary dyskinesia

| Type                        | Age (yr) | Sex | Dextrocardia | Clinical presentation |
|-----------------------------|----------|-----|--------------|----------------------|
| Total absence of outer dynein arms (Ia) | 11       | M   | -            | S, OM, BC, BE        |
|                             | 8        | F   | +            | S, BE                |
| Total absence of inner dynein arms (Ib) | 10*      | M   | -            | S, BC, BE, C         |
|                             | 12*      | M   | -            | S, OM, BC, BE        |
|                             | 12       | M   | -            | S, BC, BE, C         |
| Total absence of outer and inner dynein arms (Ic) | 13*      | F   | +            | S, OM, BC, BE        |
|                             | 15*      | M   | +            | S, BC                |
| Partial absence of outer and inner dynein arms (Id) | 6        | M   | -            | S, BC, BE           |
|                             | 10       | M   | -            | S, BC, C            |
| Total absence of radial spokes with eccentric central core (II) | 11       | F   | +            | S, OM, BC           |
| Microtubular transposition (III) | 9        | F   | -            | BC                   |
| Combined Types              |          |     |              |                      |
| Ic+III                      | 7        | M   | -            | S, OM, BC           |
| id+III                      | 10       | F   | -            | S, OM, BC, BE       |
| Id+III+III                  | 12*      | M   | -            | BC, BE              |
| Id+III                      | 14*      | F   | -            | S, BC, BE           |
| Id+III+III                  | 16*      | F   | -            | S, BC, BE           |

S: sinusitis; OM: otitis media; BC: bronchitis; BE: bronchiectasis; C: clubbing. Each symbol (#, *, /) denotes siblings.

abnormal ultrastructure was revealed on transmission electron microscopy (total absence of outer dynein arms, n=2; total absence of inner dynein arms, n=3; total absence of outer and inner dynein arms, n=2; partial absence of outer and inner dynein arms, n=2; total absence of radial spokes with eccentric central core, n=1; absence of the central pair of tubules with transposition of outer doublet to the center, n=1; total absence of outer and inner dynein arms and microtubular transposition, n=1; partial absence of outer and inner dynein arms and microtubular transposition, n=1; partial absence of outer and inner dynein arms, radial spoke defect, and microtubular transposition, n=3). All patients with recurrent infections were frequently treated with antibiotics, mucolytics, and conventional physiotherapy. All drugs were, however, withdrawn for at least two days prior to blood sampling, and all subjects were afebrile and free of acute infection at the time of the study.

Fifteen healthy subjects, recruited from the Vaccination Clinic, served as control. None had any history of recurrent infection, was taking regular medication, or had recent upper respiratory tract infection at the time of the study. The mean age of this group was 12.1 yrs (range, 3-16 yrs; M:F=8:7). Parents of all participants gave informed consent for this study, and the protocol was approved by the Hospital Ethics Committee.

Blood neutrophils were isolated by the technique described by Cerasoli et al. (11). Twenty to 30 mL of whole blood was anticoagulated with 0.1 M EDTA, then centrifuged at 300 × g for 20 min to obtain platelet-rich plasma, which was removed and centrifuged at 2,500 × g for 15 min to obtain platelet-poor plasma (PPP). This PPP was used with a Percoll™ stock solution (9 vol Percoll™: 1 vol 1.5 M NaCl) to form plasma-Percoll™ step gradients 80, 76, 62, and 51% (v/v) in Percoll™. To the erythrocyte-leukocyte pellet obtained from the first 300 × g centrifugation was added 5.0 mL of 6% dextran (mol. wt 500,000) and enough normal saline to obtain a final volume of 50 mL. The tube was mixed by inversion, and erythrocytes allowed to settle for 45-60 min. The leukocyte-rich upper layer was collected with a plastic pipette, pelleted by centrifugation (300 × g, 10 min), resuspended in autologous plasma, and applied to the plasma-Percoll™ step gradients. Cells from 20 to 30 mL of whole blood were applied to two 12 mL gradients prepared in 15 mL polypropylene tubes (15 × 125 mm), each containing 3 mL of each plasma-Percoll™ step, and centrifuged in a swinging bucket rotor at 300 × g for 10 min. Neutrophils sedimented to the top of the 76% step (occasionally of the 80%) with mononuclear cells at the top of the 51% step (occasionally of the 62%) and erythrocytes at the bottom of the tube. The purity and viability of neutrophil preparations were typically more than 95% and more than 98%, respectively.

Purified cells were washed in HEPES buffer (10 mM HEPES, 140 mM NaCl, 10 mM KCl, 0.1 mM CaCl₂, 0.2 mM MgCl₂, 11.9 mM NaHCO₃, 5.0 mM glucose, 14 mM albumin, pH 7.4) then used in chemotaxis. Neutrophil chemotaxis was performed under agarose as previously published (12). One percent aqueous agarose solution (Type 1, low EEO; Sigma) was mixed with an equal volume of 2 × concentrated HEPES buffer containing additional 2 mM CaCl₂, 2 mM MgCl₂, and 2% (wt/vol) bovine serum albumin, and 5 mL of this mixture was poured into 60-mm tissue culture dishes. After hardening, a template was used to cut wells in the agarose. Neutrophils were placed into the center well of the linear array of three wells (7 L of 35,000 cells/L), while buffer was placed in the well nearest the center of the dish and the chemotaxin in the most distant well. Cells and reagents were in HEPES buffer (10 mM HEPES, 140 mM NaCl, 10 mM KCl, 0.1 mM CaCl₂, 0.2 mM MgCl₂, 11.9 mM NaHCO₃, 5.0 mM glucose, 14 mM albumin, pH 7.4) then used in chemotaxis. Neutrophil chemotaxis was performed under agarose as previously published (12). One percent aqueous agarose solution (Type 1, low EEO; Sigma) was mixed with an equal volume of 2 × concentrated HEPES buffer containing additional 2 mM CaCl₂, 2 mM MgCl₂, and 2% (wt/vol) bovine serum albumin, and 5 mL of this mixture was poured into 60-mm tissue culture dishes. After hardening, a template was used to cut wells in the agarose. Neutrophils were placed into the center well of the linear array of three wells (7 L of 35,000 cells/L), while buffer was placed in the well nearest the center of the dish and the chemotaxin in the most distant well. Cells and reagents were in HEPES buffer containing additional 1 mM CaCl₂, 1 mM MgCl₂, and 1% bovine serum albumin. Slowly diffusible chemotaxis such as LTB₄ and C5a were put in the wells 1 hr before cells, while fMLP was placed in wells at the same time as cells (13). Cells were incubated for 2.5 hr at 37°C, fixed at 4°C with 2.5% glutaraldehyde/1.0% paraformaldehyde in Millonig’s phosphate buffer, stained with Accustain (Sigma), and the leading front measured using a Nikon inverted microscope equipped with a calibrated eyepiece reticle (14). Cells in the leading front were identified as neutrophils by standard morphologic criteria. Each grid in the reticle used was 0.25 mm. The distances that the cells migrated toward the buffer (chemokinetic distance) and toward the chemotaxin (chemotactic distance) were measured (Fig. 1). Results were expressed as both chemotactic differential (chemotactic distance minus chemokinetic distance) and
chemotactic index (chemotactic distance divided by chemokinetic distance) (15). Three to six replicates were performed for each chemotactic condition in each experiment.

Data are presented as mean ± SEM. Comparisons were made by paired or unpaired t test. A \( p \) value <0.05 was considered sufficient to reject the null hypothesis.

RESULTS

Initial experiments defined the optimum concentrations for the three chemotaxins used in this study, LTB\(_4\), C5a, and fMLP, as being 1.0 \( \mu \)M, 0.01 \( \mu \)M, and 0.1 \( \mu \)M, respectively (data not shown). The LTB\(_4\) concentration chosen is about 1,000 times greater than that found in serum or plasma of healthy subjects (16), but there are no comparable data available for C5a and fMLP. Next, in order to define the reproducibility of the chemotaxis assay, neutrophils were isolated and chemotaxis assays performed on blood samples drawn on 2 successive days from 12 volunteers. The day-to-day reproducibility for chemotactic distance and chemotactic differential for the assay was confirmed to be acceptable. Chemotaxis parameters on the second day averaged 101\% of the values obtained on the first day (range, 95 ± 4.7\% to 109 ± 7.1\%).

Chemotactic distance and chemokinetic distance in response to LTB\(_4\), C5a, and fMLP were shown in Table 2. Chemotactic distances to LTB\(_4\), C5a, and fMLP were significantly lower in blood neutrophils from the patient group than in those from the control group. Chemokinetic distances in response to the three chemotaxins were lower in blood neutrophils from the patient group, but not to a statistically significant degree.

Chemotactic response to LTB\(_4\), C5a, and fMLP was analyzed in terms of chemotactic differential (Fig. 2) and chemotactic index (Fig. 3). Chemotactic differentials in response to LTB\(_4\), C5a, and fMLP in neutrophils from the patient group were 3.18 ± 0.35, 2.29 ± 0.33, and 4.51 ± 0.30 grids, respectively; all of which were significantly lower than the corresponding values in neutrophils from the control group (3.73 ± 0.30, 2.77 ± 0.35, and 3.98 ± 0.34 grids, respectively).

![Fig. 1. Quantification of migratory functions by the leading front method. The distance, A, of cellular migration from the edge of the central well outward toward the well containing chemotaxin (C), represents chemotactic distance. The distance, D, of cellular migration from the edge of the well outward toward the well containing buffer (B), represents chemokinetic distance.](image1)

![Fig. 2. Comparison of chemotactic differential (in grids, each 0.25 mm) in response to LTB\(_4\) (10\(^{-6}\) M), C5a (10\(^{-8}\) M), and fMLP (10\(^{-7}\) M), between patients with primary ciliary dyskinesia (PCD) and controls.](image2)

![Fig. 3. Comparison of chemotactic index in response to LTB\(_4\) (10\(^{-6}\) M), C5a (10\(^{-8}\) M), and fMLP (10\(^{-7}\) M), between patients with primary ciliary dyskinesia (PCD) and controls.](image3)


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and fMLP in neutrophils from the patient group were 1.78 ± 0.12, 1.58 ± 0.09, and 2.02 ± 0.11, respectively, compared with 1.87 ± 0.11, 1.63 ± 0.12, and 2.21 ± 0.18 in neutrophils from the control group. The difference between the two groups was statistically significant for LTB₄ (p<0.05) and fMLP (p<0.01), but not for C5a (p=0.20).

DISCUSSION

We have demonstrated that neutrophils in patients with primary ciliary dyskinesia exhibit a profound inhibition in terms of their chemotactic response to LTB₄, C5a, and fMLP. The decreased chemotactic response, however, was not associated with defective spontaneous migration. The finding of a decreased chemotaxis of neutrophils from patients with primary ciliary dyskinesia suggests that the defects in neutrophil and ciliary motility may have a common structural mechanism and that the defective neutrophil migration may contribute to the increased susceptibility to infectious agents in the airways.

Evidence to suggest a role for microtubules in neutrophil migration comes from observations that drugs interfering with microtubular assembly such as colchicine reduce cell migration (17). A variety of abnormalities in the microtubule structure and arrangement in cilia and sperm tails have now been described in patients with primary ciliary dyskinesia (18). While attempts to define microtubule structural abnormalities in patient’s neutrophils that are similar to those seen in cilia and sperm tails have been unsuccessful (7), a functional study of neutrophils from the patients has shown that an integrated cytoplasmic microtubule-membrane interaction is essential for their motility (19).

The association between primary ciliary dyskinesia and defective neutrophil migration is, however, still controversial. A 13-month-old child with Kartagener’s syndrome has been reported to have marked abnormalities of neutrophil migration assessed in vivo by skin window and in vitro by capillary tube and Boyden chamber assay (8). In a study of eight children with primary ciliary dyskinesia, Pedersen et al. (20) have established that neutrophils from four patients had impaired neutrophil migration. In another study, four female patients with Kartagener’s syndrome were found to have reduced random and directed neutrophil migration as assessed by Boyden chamber assay (21). Afselius et al. (7) also reported that four of eight patients studied showed abnormally short migration distance toward serum- or bacteria-derived chemotactic factor. Canciani et al. (22) have ascertained that an impairment of neutrophil chemotaxis was a constant feature in primary ciliary dyskinesia. However, a study by Corkey et al. (9) suggested that no major disturbance exists in neutrophil function including migration. Walter et al. (23) also reported that neutrophils from patients with Kartagener’s syndrome had no significant defect in chemotaxis in response to fMLP chemotactic.

These discrepancies among the reports of neutrophil migration in patients with primary ciliary dyskinesia have not been clearly explained. It is probable that many factors influence in vitro studies of neutrophil migration. It is less likely that the observed defective motility in the present study is a phenomenon secondary to infection (24), since neutrophil migration was assessed when the patients were afebrile, free of acute infection, and not receiving antibiotic therapy. Before initiating these experiments, much consideration was given to the choice of appropriate chemotaxis assay. Physical properties of the cells such as size, stiffness, and deformability may affect their migration in a filtered assay system such as the Boyden chamber (25). In fact, Kantar et al. (19) demonstrated an increase in membrane fluidity and heterogeneity in subjects with primary ciliary dyskinesia; a result implying the possibility of overestimating cell migration in the patients as compared to normal control. We chose chemotaxis under agarose as the most suitable technique to quantify chemotaxis, since movement of the larger and/or stiffer cells is not impaired by the conditions of the assay (15). In addition, it has been reported that contaminating leukocytes do not affect neutrophil chemotaxis in this assay (13). Furthermore, with this method, both chemotaxis and spontaneous migration of neutrophils can be distinguished and measured simultaneously. In the present study, the chemotactic difference for the three chemotaxins was significantly lower in the neutrophils of the patients than in those of the controls. The chemotactic index, which would be normal if the decreased chemotactic distance was due to defective spontaneous migration (15), was also lower for LTB₄ and fMLP. This finding together with the data on chemokinetic distance, which was not statistically different, suggests that neutrophil migration is defective in chemotaxis but not in spontaneous migration. Spontaneous migration of neutrophils from patients with primary ciliary dyskinesia was found to be normal in other previous reports (7, 26). It has been suggested that cytoplasmic microtubules are not essential for leukocyte locomotion per se, but for direction-finding and leukocyte directional movement towards a concentration gradient of a chemoattractant (27).

It has been shown that defective neutrophil locomotion might be found after stimulation with one chemotaxin but normal locomotion after activation with another (24). This finding emphasizes the importance of studying neutrophils under the influence of several different chemotaxins. While a number of neutrophil chemotaxins have been identified, those chosen for this study were arachidonic acid lipooxygenation metabolite (LTB₄), a cleavage product of the fifth component of complement (C5a), and synthetic fMLP which is structurally similar to N-formyl peptides generated by bacteria, since they are representative chemotaxins, which may be generated at inflammatory sites and may serve to regulate the directed recruitment of neutrophils to inflammatory foci in vivo (28).
Our finding of decreased neutrophil chemotaxis toward the three chemotaxins examined, however, suggests that defective chemotaxis may be a universal phenomenon with regard to chemotaxis.

It is now recognized that primary ciliary dyskinesia arises in a heterogeneous group of patients in which at least six separate genetic types can be identified on the basis of ultrastructural abnormalities of microtubule in cilia and the patterns of ciliary beating (29). Table 1 shows that the patients examined in this study belong to three of these groups. The decreased chemotaxis may be related to the type of ultrastructural abnormalities. Valerius et al. (26) identified two patients belonging to type VI ciliary dyskinesia (hypermotile cilia and normal ciliary ultrastructure) as having normal chemotactic activity, whereas Canciani et al. (22) reported that a defective neutrophil chemotaxis was found in all of their patients with type V ciliary dyskinesia (abnormal cilia showing supernumerary microtubular doublets). In the present study, however, the small number of patients in each group precludes the possibility of finding statistically significant differences in neutrophil chemotaxis between the groups.

In conclusion, neutrophils from patients with primary ciliary dyskinesia showed decreased chemotaxis as compared with those from normal subjects. The data support the concept that microtubules play an important role in the regulation of neutrophil directional locomotion. Further studies to substantiate this hypothesis should therefore aim at correlating neutrophil directional migration and function during chemotaxis. Centriole, microtubule, and microfilament orientation and function during chemotaxis. J Cell Biol 1977; 75: 666-93.

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