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Neutrophil Functions in Patients with Inherited Bone Marrow Failure Syndromes

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Abstract

Background—The Inherited Bone Marrow Failure Syndromes (IBMFS) include Fanconi anemia, dyskeratosis congenita, Diamond-Blackfan anemia and Shwachman-Diamond syndrome (SDS). Previous studies reported decreased neutrophil chemotaxis in patients with SDS; there are no reports of neutrophil function in other IBMFS. In this study we examined neutrophil respiratory burst function in IBMFS patients.

Procedure—Samples from 43 IBMFS patients and 61 healthy family members were collected, shipped, and analyzed within 24 hours. We also studied samples from 12 healthy control persons immediately after collection. Neutrophils were stimulated with Phorbol 12-myristate acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (fMLP), and respiratory burst analyzed by reduction of dihydro-rhodamine and cytodrome c.

Results—There was no significant difference in the degree of fMLP or PMA-driven respiratory burst activity between each of the IBMFS subgroups and their respective family members. There was also no difference in respiratory burst activity between any IBMFS and a pooled group of all healthy family members.

Conclusions—Neutrophil respiratory burst activity from IBMFS patients does not differ from that of healthy family members.

Keywords

inherited bone marrow failure; neutrophil; respiratory burst

Introduction

The Inherited Bone Marrow Failure Syndromes (IBMFS) comprise a group of rare disorders including Fanconi anemia (FA), dyskeratosis congenita (DC), Diamond-Blackfan anemia (DBA) and Shwachman-Diamond syndrome (SDS). Despite their phenotypic heterogeneity and varying degrees of single or multilineage bone marrow failure, all IBMFS share a

significant propensity for developing myelodysplastic syndrome and/or acute myelogenous leukemia.[1-3]

Reports of immunologic and neutrophil functional status in IBMFS are limited. There are some suggestions that a few IBMFS patients exhibit various degrees of qualitative immune dysfunction. Hypogammaglobulinemia was described in 3 of 14 patients with SDS[4], 1 patient with FA[5] and in 9 of 53 patients with DC.[6] Reversal of the average CD4/CD8 ratio was reported in 10 patients with FA[7] and 4 of 6 patients with DBA[8]. Decreased percentages of circulating natural killer (NK) cells were observed in 5 of 6 patients with SDS[4] and impaired NK activity was described in 2 FA patients.[9] The immune status of patients with FA was reviewed recently.[10] Overall, the number of patients with immunodeficiency was small.

Several studies indicated that neutrophils from SDS patients had significantly impaired migratory activity. Aggett *et al.* reported decreased chemotaxis of stimulated and non-stimulated neutrophils from 14 SDS patients.[11] Ruutu *et al.* observed that neutrophils from 8 patients with SDS had normal random migration but significantly decreased chemotaxis towards casein.[12] Lithium improved chemotaxis in one patient *in vitro* and *in vivo*. [13] Kuijpers *et al.* showed that neutrophils from 13 of 20 SDS patients had on average 65% lower chemotactic activity than those from unrelated controls.[14] Stepanovic *et al.* observed that neutrophils from 14 patients with SDS responded normally to pulsed gradients of fMLP, however, they exhibited neither orientation nor chemotaxis towards a spatial fMLP gradient. [15]

There are reports of abnormal bactericidal activity in neutrophils from a few patients with FA and SDS. Korkina *et al.* showed that neutrophils from 9 FA patients had a higher degree of luminol reduction than laboratory controls.[16] However, neutrophils from SDS patients were found to have normal respiratory burst and phagocytic activity.[14] Hematopoiesis in FA appears to be exquisitely sensitive to exogenous and endogenous oxidative stress, and protein products of multiple FA genes have been shown to modulate the repair of DNA damage induced by reactive oxygen species (ROS; reviewed in [17]). Since neutrophils are important generators of oxidative stress and there are a few reports of impaired neutrophil motility in SDS patients, we hypothesized that IBMFS patients might have altered neutrophil respiratory function. We therefore investigated neutrophil respiratory function in samples from patients with FA, SDS, DBA and DC, as well as samples from healthy family members subjected to the same conditions of collection, transportation and functional analysis.

Material and Methods

Study population

We studied samples from 43 IBMFS patients (11 FA; 18 DC; 4 SDS; 10 DBA) and 56 healthy family members (13 FA; 28 DC; 8 SDS; 7 DBA) from the National Cancer Institute IBMFS cohort. The study was approved by the Institutional Review Board of the NCI [NCI Protocol 02-C-0052; NCT-00342537]. Neutrophil function assays were also performed on fresh samples from 12 healthy laboratory controls at the test site (Toronto, Canada).

Sample collection and shipping

Five ml of blood was collected in EDTA at the NIH Clinical Center in Bethesda MD. Samples from patients and healthy family members were shipped overnight on wet ice to Toronto, Canada, and analyzed within 24 hours from collection.

Neutrophil purification

Five ml blood was added to 4 ml one-step Polymorph Prep (Accurate Chemical & Scientific CORP, Westbury, NY) and centrifuged at 1600 rpm for 30 minutes. The bottom layer was transferred to a new 15 ml tube, and the neutrophils were washed with Phosphate Buffered Saline (PBS), and counted in a hemocytometer.

Cytochrome C assay

A mixture of 0.88 ml PiCM-G buffer (138 mM NaCl, 2.7 mM KCl, 0.6 mM CaCl₂, 1.0 mM MgCl₂, 5mM glucose, 10 mM NaH₂PO₄/Na₂HPO₄, pH 7.4), 0.1 ml (1×10^6) neutrophils and 10 μ l (0.1 mM final concentration) was placed in a cuvette. The reference cuvettes were prepared in the same fashion, replacing 10 ml of the PiCM-G buffer with 10 ml (total 100 μ g) of SOD (bovine erythrocyte Superoxide Dismutase). Cuvettes were then incubated at 37°C for 10 minutes, and stimulated for 5 minutes with 10 μ l fMLP (N-formyl-methionyl-leucyl-phenylalanine, Sigma St-Louis, MO, 10^{-6} M final concentration) or PMA (Phorbol 12-myristate acetate, Sigma St-Louis, MO, 10^{-5} M final concentration). Absorbance of reduced cytochrome C was measured at 550 nm.[18, 19]

DHR assay

Neutrophils (1×10^6) were suspended in a solution containing 500 μ l of PBS and 2 μ M DHR-123 (Dihydro-Rhodamine, Sigma, St-Louis, MO). The mixture was incubated at 37°C for 20 minutes, then stimulated with fMLP (10^{-6} M final concentration) or PMA (10^{-5} M final concentration) for 10 minutes at 37°C; fluorescence at 530 nm was quantified by flow cytometry. [20, 21]

Statistical analysis

Ratios of results from stimulated/unstimulated neutrophils were calculated. Two-sided, unpaired Student t-tests were used to compare different methods of detecting ROS generation; $p < 0.05$ was significant. Excel and Stata 10 were used for the analyses.

Results

Figure 1 shows the ratios of reduction of DHR and cytochrome C by stimulated/unstimulated neutrophils from IBMFS patients and their unaffected first-degree relatives. Reduction of DHR was used to assay the generation of ROS in 30 patients and 45 healthy family members. Each sample was independently stimulated with fMLP (panel A) and PMA (panel B). Similarly, cytochrome C reduction was used to assay the generation of ROS in 13 patients and 16 healthy family members. Samples from SDS patients and family members as well as DBA family members were not available for this analysis. Only fMLP-stimulated neutrophils from DBA patients had a significantly increased degree of DHR reduction compared with their healthy family controls ($p = 0.04$) and compared with neutrophils from FA patients (Figure 1A, $p = 0.03$).

None of the healthy family members exhibited a statistically significant difference in ROS generation. We therefore combined all the healthy family member data and tested each IBMFS subgroup against this larger control group (Figure 2). This analysis failed to reveal any significant difference between any of the IBMFS groups and the controls. The significantly more extensive reduction of DHR by fMLP-stimulated neutrophils previously seen in DBA patients (Figure 1A) compared with their family controls was not sustained in the combined analysis (Figure 2A). The statistical significance in the DBA group was conferred by 3 affected members of the same family who all had high level of reduction of DHR. There were however no healthy members available for comparison.

In order to investigate whether shipping and delay between sampling and analysis had an effect on neutrophil respiratory function, we compared 12 fresh samples from healthy laboratory controls with our shipped healthy family member samples. The neutrophils from these family members were significantly less active in the DHR assays than the fresh laboratory specimens ($p < 0.001$) (Table I), but were within the range of the fresh samples in the cytochrome C assays.

Discussion

We investigated neutrophil respiratory function in IBMFS patients and healthy family member controls subject to the same conditions of collection, transportation and functional analysis. We used two neutrophil activators (PMA and fMLP) and analyzed respiratory burst activity by measuring the degree of reduction of DHR and cytochrome C. Since our patient and healthy family member samples were subjected to the same conditions of transportation and analysis, we expected to detect differences in neutrophil function between the two groups, albeit possibly attenuated by shipping. However, we did not identify any significant difference in the degree of respiratory burst between neutrophils from IBMFS patients and healthy family members. This is consistent with Kuijpers *et al.* who showed normal phagocytosis and NADPH-oxidase activity in SDS neutrophils. [14] In contrast with the increased burst activity reported by Korkina *et al.* in patients with FA, the neutrophil activity in our patients with FA was similar to the activity in healthy family members. Methodologic differences make the comparison difficult, as Korkina *et al.* used concanavalin A as a stimulator and measured respiratory burst by the reduction of luminol, while we used fMLP and PMA as stimulants and measured reduction of DHR and cytochrome C.

In addition to our null results in FA and SDS, we also did not identify any significant difference in the degree of neutrophil activation of patients with DBA and DC compared to healthy family controls. Overall, the relatively small number of study samples might have reduced our statistical power to detect a difference in activity. It is also possible that there is indeed no abnormality in neutrophil function in any of the IBMFS patients. Although neutrophil function may be affected by transportation, the shipped controls were essentially normal in the cytochrome C assay. Despite the fact that chemotaxis has been shown to be abnormal in some SDS patients [11, 12, 15], we did not investigate neutrophil chemotaxis because of the known unreliability of results for this test on shipped samples.

A strength of our study is that we analyzed samples from the four most frequent rare IBMFS and their healthy family members. A limitation is that all of our samples were shipped, which may have compromised neutrophil function. We could invoke a differential shipping-related functional decay, in which samples from patients were affected more than samples from healthy family members, but this seems unlikely. However, since all of our samples were subjected to the same conditions of transportation, it is possible that the null results of neutrophil respiratory burst activity reported in this study are, in fact, valid.

Our study represents the first attempt to analyze neutrophil respiratory activity in all categories of patients with IBMFS from a single cohort. Although it is well known that neutrophil function should be analyzed only on fresh samples, many publications do not explicitly state that samples analyzed were indeed fresh. The decrease of neutrophil activity in some assays related to transportation may constitute a barrier to performing collaborative, multi-site neutrophil function studies using a single standardized reference laboratory.

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Figure 1.

Respiratory burst activity in neutrophils from IBMFS patients and from their healthy family member controls. Horizontal axis: Patients (DHR assay: 8 FA, 11 DC, 7 DBA, 4 SDS; CytoC assay: 3 FA, 7 DC, 3 DBA, 0 SDS), Controls (DHR assay: 13 FAC, 15 DCC, 9 DBAC, 8 SDSC; CytoC assay: 3 FAC, 13 DCC; 0 DBAC; 0 SDSC). Vertical axis: ratios of DHR and cyto C reduction by stimulated/unstimulated neutrophils. Panels A and C) fMLP; B and D) PMA; A and B) DHR; C and D) CytoC. $p=0.04$ for DBA patients vs. DBA healthy family members in the DHR fMLP assay & $p=0.03$ for FA patients vs. DBA patients in the fMLP assay

Figure 2.

Respiratory burst activity in neutrophils from IBMFS patients and pooled healthy family member controls. Horizontal axis: Patients (as in figure 2), Controls (pooled healthy family members from figure 2). Vertical axis: ratios of DHR and cyto C reduction by stimulated/unstimulated neutrophils A and B) fMLP; C and D) PMA; A and C) DHR; B and D) CytoC

Table I
Respiratory Burst Activity in Shipped vs Fresh Control Neutrophils*

Assay	Shipped family members*	Fresh Laboratory Controls	Shipped as % of Fresh	p
DHR (fMLP)	1.52 ± 0.75 (45)	5.14 ± 2.98 (12)	28	<0.001
DHR (PMA)	3.00 ± 2.58 (43)	15.09 ± 4.09 (12)	20	<0.001
CytoC (fMLP)	2.85 ± 1.74 (16)	3.97 ± 2.37 (12)	70	0.16
CytoC (PMA)	9.7 ± 10 (16)	15.33 ± 5.74 (12)	63	0.09

* Mean ratios (± 1 SD) of DHR reduction. Number of samples in each assay is in parentheses.