Quantitative and volume, conductivity and scatter changes in leucocytes of patients with acute undifferentiated febrile illness: a pilot study

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Background: A single diagnostic test for acute undifferentiated febrile illnesses (AUI) is elusive. This pilot study was undertaken on the premise that leucocytes, being the main cells of defence, undergo quantitative, structural and functional changes in AUI. We evaluated the potential of volume, conductivity and scatter (VCS) parameters of leucocytes, generated with the haemogram report by the Coulter auto-analyzer, in differentiating the common etiologies of AUI.

Methods: The haematological and VCS data obtained from 800 controls and 200 cases of AUI (50 cases each of acute malaria, dengue, scrub typhus and enteric fever) were retrieved for analysis.

Results: The cases and controls differed significantly with respect to relative numbers and the VCS parameters of neutrophils, lymphocytes and monocytes ($p < 0.05$). The neutrophil and lymphocyte were significantly voluminous in acute malaria and scrub typhus as compared to dengue and enteric fevers ($p < 0.05$). Enteric fever significantly enhanced the conductivity of neutrophils as compared to other subgroups while lymphocyte conductivity significantly differed from dengue and scrub typhus. Lymphocyte and neutrophil scatter values in malaria and scrub typhus were comparable but differed significantly from that in enteric fever.

Conclusions: Etiology-specific changes occur in leucocytes, both in numbers and their VCS properties, which can be identified without additional cost.

Keywords: Acute undifferentiated febrile illness, Coulter, India, Tropical infections, VCS technology

Introduction

Acute undifferentiated febrile illness (AUI) is defined as acute onset fever ($>38^{\circ}\text{C}$) of less than 2 weeks duration without an apparent cause despite a meticulous history and physical examination. In the developing world, the differential diagnosis for AUI includes significant illnesses, (such as bacterial sepsis, malaria, dengue fever, enteric fever, leptospirosis, rickettsiosis and Japanese encephalitis) both in terms of mortality and morbidity.¹ The clinical symptoms and signs overlap significantly; therefore, diagnosing the specific etiology of AUI can be difficult, incorrect and time-consuming precluding appropriate patient management.

The interpretation of the tests commonly ordered in this scenario, namely complete blood counts, peripheral blood smear examination, cultures of available specimens and serological

tests, suffer major pitfalls. Moreover, variable sensitivities and specificities of tests, lack of proper collection and storage techniques, non-availability of technical expertise and/or infrastructure for different tests and the delay in obtaining the results pose further limitations to proper diagnosis.² This often leads to erroneous initiation of empirical antibiotic therapy thereby promoting antimicrobial resistance.

Infections, due to different etiological agents, are associated with an alteration of white cell counts. Some morphological and functional changes also occur in the leucocytes during infections; however, they have not been well characterized. More and more modern laboratories are using the automated hematology analyzer that can readily obtain the total and differential leucocyte count, almost universally ordered in this scenario. The volume, conductivity and scatter (VCS)

technology of the Coulter LH 750 hematology analyzer (Beckman Coulter, Fullerton, CA, USA) can obtain data from more than 8000 WBCs. It uses direct current impedance to measure cell volume (V) for accurate size of all cell types, radio frequency opacity to characterize conductivity (C) for internal composition of each cell, and a laser beam to measure light scatter (S) for cytoplasmic granularity and nuclear structure. The data are used to identify each cell as a neutrophil, lymphocyte, monocyte, eosinophil or basophil, generating an automated differential count. Band forms and other immature granulocytes (metamyelocytes, myelocytes), as well as reactive segmented neutrophils, tend to be larger and have lower nuclear complexity than their normal inactivated counterparts. Therefore, the morphologic changes seen in the left-shifted and reactive segmented neutrophils could be analyzed quantitatively by using the Coulter LH 750 with VCS technology.³

The VCS technology has been used to evaluate morphologic changes within the same cell population, such as the changes occurring in neutrophils during acute bacterial infection. Celik et al.⁴ studied the levels of neutrophil VCS parameters in neonatal sepsis and observed significant increases in mean neutrophil volume (NV), neutrophil volume distribution width (NVDW), neutrophil conductivity distribution width (NCDW), and significant decreases in mean neutrophil conductivity (NC) and mean neutrophil scatter (NS). Significant decreases were observed in mean NV, NVDW, and NCDW, whereas mean NC and NS increased at the end of the treatment. Gram-negative sepsis caused higher mean NV and NVDW than Gram-positive sepsis.⁴ Lee and Kim⁵ evaluated mean cell volumes of neutrophils and monocytes in elderly patients with sepsis. They observed that the mean NV and monocytes volume (MV) were higher in the sepsis group than in the localized infection and control groups ($p < 0.001$ for both). A mean NV of 156.5 fL or higher was suggested as a predictor of sepsis with a high sensitivity (83.3%) and specificity (78%).⁵

Besides bacterial infections, literature also exists on VCS technology in viral infections. Koenig and Quillen demonstrated higher NVDW in bacterial infections and higher lymphocyte volume distribution width (LVDW) in viral infections in childhood.⁶ Similarly, the lymph index, derived from the VCS parameters, was observed to be significantly increased in viral infections when compared to acute bacterial infections and controls in the study from China.⁷ Attempts have also been made to differentiate malaria and dengue fevers on the basis of VCS technology.

However, literature for other causes of fever, like rickettsial diseases, enteric fever and TB, does not exist. We postulated that irrespective of their etiologies, febrile illnesses produce changes in leucocytes both in terms of numbers and properties. Further, different etiological agents of AEFI, namely, malaria, dengue, scrub typhus and enteric fever, induce specific changes in the leucocytes. We undertook this pilot study to assess the changes in the numbers and VCS parameters of activated leukocytes in patients with AEFIs due to acute malaria, dengue, scrub typhus and enteric fever, a major reason for the population in this part of the world to seek medical care.

Materials and methods

Study setting

Uttarakhand is a mountainous state in northern India, the Ganges being its major river system. The varied vegetation includes alpine meadows, subalpine conifer and subtropical pine forests, moist deciduous forests and grasslands. Nearly 70% of its 10 million population resides in rural areas. Uttarakhand has two major divisions: Garhwal comprising seven north-western districts and the Kumaon division comprising six districts. TB is the major infectious disease; tropical diseases, namely, leishmaniasis⁸ and scrub typhus⁹ have been reported from the region only recently. There are regular outbreaks of AEFI mostly during and after monsoons. The Himalayan Hospital is a 1000 bed tertiary care teaching hospital affiliated to the Himalayan Institute of Medical Sciences located 25 km from Dehradun, the capital of Uttarakhand. The hospital mainly caters to the Garhwal division, some districts of the Kumaon division and the densely populated adjoining districts of Uttar Pradesh.

Patient selection

This observational pilot case-control study was undertaken on 200 adult patients with an established diagnosis of acute malaria, dengue fever, scrub typhus or enteric fever ($n=50$ in each subgroup) presenting with acute fever of less than 15 days duration. Eight-hundred blood samples of apparently healthy university staff (400 males) collected during their annual check-up were used as controls. The data was anonymised and institutional ethical clearance was obtained.

Malaria was diagnosed by smear positivity, dengue infection by positive IgM serology (rapid card test-SD Bioline) while scrub typhus was diagnosed by positive IgM ELISA. Enteric fever was diagnosed on the basis of culture positivity for *Salmonella* sp., and/or positive Widal test (defined as somatic O and flagellar H titre >160 at baseline or a fourfold rise in titre over 2 weeks). Those with co-infections and/or with underlying chronic diseases like arthritis, malignancy, hypothyroidism and chronic kidney disease were excluded.

Data collection

The demographic, clinical and laboratory data of all those included was compiled from the hospital records. The VCS data obtained from blood samples drawn at the time of presentation to the hospital and analysed by the Coulter counter LH750 was retrieved for analysis.

Data analysis

Mean was used as the measure of central tendency and standard deviation as the measure of dispersion for descriptive statistics. Analysis of variance (ANOVA) with Bonferroni post-hoc analysis was used to compare the means of various etiological groups using the statistical software SPSS version 19 (IBM, Armonk, NY, USA). A p -value of <0.05 was considered as significant.

Results

In this study, the patients with acute undifferentiated fever (n=200) presented to our hospital after an average of 7.4 days. The mean age of patients was 38.5±15 years with male predominance. Malaise (70/200; 35.0%) and abdominal pain (63/200; 31.5%) were the predominant complaints. Other abdominal complaints were vomiting (51/200; 25.5%), loose stools (44/200; 22.0%) and nausea (26/200; 13.0%). Hepatomegaly (127/200; 53.5%), splenomegaly (61/200; 30.5%) and lymphadenopathy (10/200; 5.0%) were the important clinical signs elicited in these patients.

Although the means of haemoglobin, red cell and platelet counts revealed statistically significant differences between the cases and controls, the averages of the total leucocyte counts, and mean red corpuscular volume, haemoglobin, and its concentration were comparable. The white cell differential and their VCS parameters were significantly different (p<0.001) in comparison to the controls (see Table 1).

The cases were sub-categorised into the specific etiologic groups and the leucocyte characteristics were re-analysed in relation to controls and the other etiological groups. While significant reduction in total leucocyte count was observed in those with malaria as compared to controls, scrub and enteric fever, leucocytosis was seen prominently in scrub typhus.

Similarly, monocytosis was conspicuously observed in dengue. While eosinopenia was noticed in malaria, dengue and scrub typhus in relation to the controls, those with dengue had significant neutropenia as compared to the other etiologic groups.

The mean NV, MV and lymphocyte volume (LV) increased significantly in all patients with fever over controls. NV and LV were significantly more in malaria and scrub typhus when compared to dengue and enteric fever. Although higher than controls, the mean MV was significantly lower in enteric fever compared to the other etiological groups (p<0.05). A raised mean NC and a reduced mean monocyte conductivity (MC) was observed in all sub-groups over controls. The mean distribution width of leucocyte volumes, conductivity and scatter were significantly increased in all subgroups of patients, however, the rise was least in those with enteric fever (see Table 1).

Discussion

Activated leucocytes undergo changes both in terms of relative numbers as well as their properties in febrile illnesses proving that these are the main cells responsible for the body's defense. Contrary to the popular belief that polymorphs and lymphocytes

Table 1. Comparison of the total and differential leucocyte counts and VCS characteristics of AUI based on the etiology

Parameter	Controls (n=800)	AUI (n=200)	Malaria (n=50)	Dengue (n=50)	Scrub typhus (n=50)	Enteric fever (n=50)
Total leucocyte count (x10 ³ /cumm)	8.0±1.9 ^{M,S,D}	8.1±5.0	5.9±3.2 ^{S,E}	6.4±4.2 ^S	11.5±6.6 ^E	8.6±2.7
Neutrophil (%)	61.2±9.4 ^{A,M,S,D}	64.1±16.8	67.3±15.0 ^D	52.9±18.2 ^{S,E}	71.2±15.3	65.3±11.4
Lymphocytes (%)	28.1±7.8 ^{A,M,S,E}	23.9±12.7	22.7±14.0	29.1±11.1	21.3±13.7	22.7±9.4
Monocyte (%)	7.1±1.9 ^{A,M,D}	9.9±7.8	8.9±6.9 ^D	16.1±10.4 ^{S,E}	6.4±4.2	8.1±3.3
Eosinophil (%)	2.9±3.2 ^{A,M,S,D}	1.4±2.5	0.7±1.0 ^E	1.2±0.5 ^E	0.6±1.1 ^E	3.2±3.9
Neutrophil volume (NV)	147.4±5.8 ^{A,M,S,D,E}	160.9±15.1	167.3±14.6 ^{D,E}	153.6±9.8 ^S	168.5±14.7 ^E	154.1±13.1
Neutrophil conductivity (NC)	142.4±8.8 ^{A,M,S,D,E}	151.3±8.9	152.0±6.3 ^{D,E}	147.2±8.4 ^E	148.6±8.7 ^E	157.5±7.8
Neutrophil scatter (NS)	153.7±9.2 ^{A,M,S,D,E}	134.9±8.5	132.9±9.2 ^{D,E}	138.2±5.7 ^S	131.1±8.2 ^E	137.3±8.2
NVSD	19.8±1.4 ^{A,M,S,D,E}	26.6±5.8	29.1±5.9 ^{D,E}	26.6±5.4 ^{S,E}	28.6±5.8 ^E	22.1±2.9
NCSD	5.6±0.9 ^{A,M,S,D,E}	8.2±2.5	8.9±2.3 ^{D,E}	8.0±1.8 ^{S,E}	9.3±3.2 ^E	6.8±1.5
NSSD	10.6±1.7 ^{A,M,S,D}	13.6±3.0	13.3±2.0 ^{D,E}	16.0±3.1 ^{S,E}	13.9±2.9 ^E	11.3±1.8
Monocyte volume (MV)	166.9±5.4 ^{A,M,S,D,E}	184.3±14.1	193.8±12.8 ^{D,S,E}	184.0±11.5 ^E	185.8±13.2 ^E	173.7±10.2
Monocyte conductivity (MC)	85.7±8.8 ^{A,M,S,D,E}	83.5±7.1	85.1±6.9	84.2±5.5 ⁰	81.1±8.1	83.5±6.9
Monocyte scatter (MS)	128.6±7.6 ^{A,M,S,D,E}	128.2±8.4	130.5±6.5 ^S	126.2±7.6 ^E	123.9±9.0 ^E	132.3±7.3
MVSD	17.8±1.8 ^{A,M,S,D,E}	25.9±5.3	28.3±4.7 ^E	26.9±3.9 ^E	26.9±5.4 ^E	21.7±4.5
MCSD	4.7±0.7 ^{A,M,S,D,E}	6.3±2.3	6.7±2.8 ^E	6.2±2.1 ^E	6.5±2.5 ^E	5.5±1.3
MSSD	11.4±2.7 ^{A,S,D}	13.4±3.7	12.3±2.8 ^D	16.5±3.6 ^{S,E}	13.1±3.4	11.9±3.0
Lymphocyte volume (LV)	83.2±2.9 ^{A,M,S,D,E}	92.7±9.0	97.7±6.3 ^{D,E}	87.5±6.8 ^S	98.4±9.5 ^E	87.4±5.8
Lymphocyte conductivity (LC)	120.0±7.6 ^{A,M,E}	122.4±8.1	124.8±6.6 ^D	118.9±7.8 ^E	120.7±9.0 ^E	125.4±6.7
Lymphocyte scatter (LS)	61.9±10.8 ^{A,M,S}	66.4±10.5	70.9±11.3 ^E	64.9±8.6	68.1±8.9 ^E	61.7±10.2
LVSD	14.1±1.2 ^{A,M,S,D,E}	18.2±3.6	18.0±3.2 ^{S,E}	18.6±3.0 ^E	19.6±4.7 ^E	16.7±2.9
LCSD	10.4±2.4 ^{A,M,S,D,E}	12.4±3.5	12.1±3.2	12.9±3.2	12.8±4.2	11.9±3.3
LSSD	18.0±3.3 ^{A,M,S,D}	21.5±4.2	20.1±3.4 ^{D,S}	23.5±3.0 ^E	23.1±4.9 ^E	19.4±3.7

AUI: acute undifferentiated febrile illnesses; SD: standard deviation/distribution width; VCS: volume, conductivity and scatter.

Significantly different from ^A AUI, ^M malaria, ^D dengue, ^S scrub typhus, ^E enteric fever (ANOVA and Bonferroni post hoc analysis; p<0.05).

undergo changes in different type of infections, we observed that almost all leucocytes undergo morphological changes and presumably in terms of their properties. Also, these changes appear to be specific to the type of leucocyte and the VCS properties are relatively specific to the etiological agent producing the febrile illness.

The immune-pathogenesis of the etiological agents of AUFI is partially and poorly understood and represents a complex interplay of adaptive, humoral and cell-mediated immunity with interleukins, cytokines and a variety of specific and non-specific receptors. The impact of these infectious agents on the leucocytes and vice-versa have not been well characterized. Acute malarial infection induces immediate, non-specific and poorly defined humoral and cellular immune response that tends to limit the progression of disease. Also, this immunity lasts for a short period in the absence of re-exposure.¹⁰ The monocytes, macrophages, and dendritic cells are the major phagocytic cells of the innate immune system, and are responsible for detecting and removing invasive pathogens in dengue fever. They are also the antigen presenting cells critical for the initiation, expansion, and polarization of adaptive cellular immunity. Targeting these cells by the dengue virus has a significant impact on immune modulation.¹¹ Endothelial cells are mainly infected by *Orientia tsutsugamushi* though the organisms may be found in several other cells, including dendritic cells, macrophages, polymorphs and lymphocytes. Uptake and ensuing intracytoplasmic destruction of the bacterium is facilitated by both cellular and humoral immunity. Identification of protective antigens is important to understand homotypic and heterotypic immunity to scrub typhus, however, the molecules that partake protective immunity are partially known.¹²

During the active infectious disease state and when the patient is febrile, there is an increase in the volumes of polymorphs, lymphocytes as well as the monocytes. From our study, it is apparent that monocytes swelled the most in malarial infection in comparison to other infectious etiologies studied. Scrub typhus and malaria induced comparable but significant increase in the volumes of the neutrophils and lymphocytes when compared to dengue or rickettsial infection. The internal composition of the leucocytes, especially the neutrophils and lymphocytes, (assessed by conductivity) were rendered significantly opaque to the radiofrequency in enteric fever compared to the controls and other etiologies. The cytoplasmic granularity and the nuclear structure of the lymphocytes (assessed by the scatter values) were significantly altered in enteric fever over other febrile illnesses but were comparable to the controls, despite the changes in the conductivity. Volumes of neutrophils, monocytes and lymphocytes were increased in acute malaria; additionally, conductivity of monocytes and lymphocytes is altered. Lymphocyte scatter (LS) was also significantly increased in malaria. The neutrophil and lymphocyte volumes as well as conductivities were reduced significantly in those with dengue fever in our study. Additionally, dengue infection had a significant impact on neutrophil scatter compared to non-dengue fevers. Enteric fever increased the volumes of leucocytes but reduced their conductivity. Variable changes in volume and other properties of leucocytes were observed in scrub typhus when compared to those with non-scrub AUFI.

Due to the considerable overlap in the clinical features and the hematological and biochemical variables, the confirmation of the etiology entails numerous diagnostic tests that are both time consuming and costly. The present study provides insight

into the potential of the VCS properties of leucocytes in differentiating the main causes of acute febrile illnesses in this part of the world. This additional information is available at no extra cost as it is generated as part of the auto-analyzer haemogram report and is usually discarded.

Earlier, it was contested that there is a significant variation in the VCS parameters of the leucocytes even in the healthy individuals. Tang et al.¹³ in 2012 demonstrated little fluctuation in biological variations for cell population data in healthy individuals at homeostatic set point. Thus, this makes them reasonably reliable parameters clinically.¹³ Some work on the changes in VCS parameters has been undertaken in patients with acute malaria. In a study conducted by Briggs et al.,¹⁴ response to malarial infection was shown to cause an increased monocyte count and production of large activated monocytes detectable by the VCS technology. We also observed a significant increase in the mean MV as well as in their relative quantity in malarial infection. By using a calculation derived from the SD of the volume of the lymphocytes and monocytes, a malaria factor was put forth for the detection of malaria with a very high sensitivity (98%) and specificity (94%). Compared to the controls, the mean NV, LV, MV and monocyte scatter (MS) were increased, and NS and MC were reduced in malaria in the study by Briggs et al.¹⁴ and the present study. However, the pattern of NC, lymphocyte conductivity (LC) and LS was paradoxical in the two studies and there was a disparity in the absolute values in the control group as well. This could be attributed to racial and, possibly, environmental differences in the two populations studied. Also, the values put forth in the study by Briggs et al.¹⁴ are of a single patient of falciparum malaria. It is not a scientific comparison, nonetheless, it gives an insight into the alteration of VCS parameters in malaria, given the paucity of literature on the subject.

Sharma et al.¹⁵ very recently quantitated leucocyte abnormalities by automated analyzers to successfully identify malaria and dengue and distinguish them from other fevers. The mean values of NS, MV, MS, monocyte scatter distribution width (MSDW), and LC of our control group were higher and those of NVDW, NCDW, MC, monocyte conductivity distribution width (MCDW), LV and LS were low as compared to the controls mentioned by Sharma et al.¹⁵ This appears to be due to the difference in the controls in the two studies. While Sharma et al. used the values of febrile controls, we took apparently healthy controls. Barring MS and LC that reduced in malaria in the earlier study in contrast to the present study, the remaining parameters showed a similar trend of rise and fall in the two studies.

Reliance on serological tests for the diagnosis of dengue and scrub typhus was the major limitation of the present study. Also, cases of malaria were clubbed together, irrespective of the infecting species, similar to the methodology followed by the earlier studies. Moreover, exclusion criteria were fulfilled mainly on clinical grounds, leaving scope for the relative impact of the chronic diseases on the VCS parameters. The controls belonged mainly to the 22–45 year age group, hence, the values of the controls might not be applicable to the general population. Nevertheless, as most of our patients also belonged to the same age group, this limitation was overcome to a large extent for the present study.

How these changes in VCS of leucocytes translate into functional changes is unclear. Also, whether these changes are

dependent on the virulence of the organism and/or the immune status of the host remains to be seen. A diagnosis (with a certain degree of specificity) could be based on the clinical features and a single blood sample analysis by VCS machine available in various modern labs. The cost of the machine is prohibitively high, yet it has the potential to avoid unnecessary investigations, thereby curtailing the time and cost of arriving at a definitive diagnosis. This may have significant ramifications in decreasing the morbidity and mortality associated with the delay in initiation of specific treatment for AUFI.

We are working further on the specific etiological groups on large number of subjects and shall soon present the cut-off values that will aid in the diagnosis of the etiology of AUFI with high sensitivity and specificity without any extra financial burden or technical expertise. These automated discriminant functions can be rapidly calculated by analyzer software programs to generate electronic flags to trigger specific testing. This could potentially transform diagnostic approaches to tropical febrile illnesses in cost-constrained settings.

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