Original Article

Lymphocyte Subset Alterations in Childhood Iron Deficiency Anemia: A Case–Control Study from Northern India

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Abstract

Background: The impact of iron deficiency anemia (IDA) on innate immunity is well documented, but the data on its effect on cells of the adaptive immune system are scant. The aim of this study was to assess peripheral blood lymphocyte subsets in children with IDA using flow cytometry. **Materials and Methods:** The total and differential lymphocyte populations of 80 iron-deficient children and 40 controls were assessed using a single-tube flow cytometry panel. **Results:** Children in the IDA group showed a significant decrease in total leukocyte count as compared to controls ($P = 0.03$). The study also found a significant increase in total lymphocytes and CD19 + B cells ($P = 0.042$ and 0.040, respectively). CD3, CD4, CD8, and NK cells were unaltered. **Conclusion:** The study concluded that iron deficiency in children is associated with relative lymphocytosis contributed mainly by the B lymphocytes. There is no quantitative defect in cells of the adaptive limb of the immune system in IDA. In view of the increased incidence of infections in these children, functional assays for identifying qualitative defects may prove useful.

Keywords: CD4, CD8, children, iron deficiency anemia, lymphocyte subsets

Introduction

Iron deficiency anemia (IDA) is one of the most prevalent micronutrient deficiencies, especially in developing countries. A survey by the World Health Organization reported a 59% prevalence of anemia among Indian preschool children, iron deficiency being the most common cause.[1] Iron deficiency has manifold adverse impacts on health including an increased susceptibility to common infections which may be attributed to a weakened immune system in these children.[2,3]

It is well known that neutrophil functions including phagocytosis and oxidative burst are impaired due to iron deficiency. Comparatively, the effect of IDA on the adaptive immune system is not well understood. Some studies have found an increase in the total lymphocyte count in association with iron deficiency in children, but this is not validated by other authors.[4-8] Similarly, the effect of iron deficiency on the total number of B cells, T cells, and different subpopulations is also not well documented. The knowledge of the effect of iron deficiency on effector cells may help us in understanding the nature of infection these children may be prone to. Thus, the aim of this study was to assess the percentage of peripheral blood lymphocyte populations in iron-deficient children by flow cytometric assay and to compare the data with healthy age- and sex-matched children.

Materials and Methods

The study was conducted after obtaining clearance from the institutional ethics committee (ethical clearance certificate number – F.17/IEC/MAMC/2017/Path/05 dated October 27, 2017). Children between 1 and 12 years of age presenting to the pediatrics outpatient department were enrolled for this study. A total of 80 cases and 40 controls were selected. Consent was taken from all subjects.

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Inclusion criteria included

Hemoglobin (Hb) levels below 2 standard deviations for age, mean corpuscular volume (MCV) <80 fL, serum ferritin levels below 7 ng/ml and/or transferrin saturation <10%. Children with albumin levels <3.5 g/dl, history of recent acute blood loss, dimorphic anemia, severe acute malnutrition, history of receiving iron replacement therapy in the last 3 months, history of intake of immunosuppressants, radiotherapy, or chemotherapy in the last 1 year were excluded. Diagnosed cases of autoimmune diseases, chronic illness (renal, hepatic illness), or malignancy were also excluded. Children in the control group were healthy, matched for age and sex, and had Hb, MCV, and serum ferritin levels within the normal range for age.

Sampling

Two milliliters of venous blood was withdrawn in K3-EDTA for complete blood counts (CBC), peripheral blood smears (PBS), and flow cytometric analysis. In addition, 3 ml of blood was collected in a plain tube (no anticoagulant), left to clot and centrifuged at 5000 rpm for 5 min. The serum was then separated and stored at − 20°C till used for serum iron, serum ferritin, total iron-binding capacity (TIBC), and serum albumin.

Laboratory analysis

CBC was performed on automated hematology analyzer (Sysmex XT-1800i). Hb, total leukocyte count (TLC), MCV, hematocrit, platelet count, and other RBC parameters were assessed and noted. PBS was made according to the standard guidelines using Giemsa stain and the degree of microcytosis and hypochromia graded as mild, moderate, and severe. Flow cytometric analysis was performed on FC500 Beckman and Coulter dual laser five color instrument. Single-tube flow analysis was done as follows: CD4-FITC, CD8-PE, CD3-ECD, CD45-PE-Cy5, and CD19-PE-Cy7. The analysis was done using SPSS (IBM SPSS Statistics pvt ltd, USA) software version 17. Debris was separated using viability gate. Gating strategy CD45 versus side scatter was used to separate lymphocytes and monocytes. T and B lymphocyte populations were further separated using antibodies for CD3 and CD19. The CD3 positive T cells were stained using CD4 and CD8 surface markers, and subtyping into T helper cells (CD4+/CD3+) and cytotoxic T cells (CD8+/CD3+) was done [Figures 1 and 2].

Biochemical analysis

Biochemical analysis was performed on a fully automated clinical chemistry analyzer (Randox Imola) and included serum ferritin (ng/dl) measured by electrochemiluminescence; serum albumin (g/dl), serum iron (mcg/dl), and TIBC (mcg/dl) measured by spectrophotometry. Transferrin saturation (%) was calculated using the formula: serum iron/total iron-binding capacity × 100.

Statistical analysis

Statistical analysis was performed using SPSS software version 17. The outcome variables were expressed in means and medians and the difference between the mean and median serum values of all the outcome variables was calculated using the students independent *t*-test or Mann–Whitney-U test. $P < 0.05$ was taken as statistically significant. Linear regression analysis was done using the Spearman correlation test.

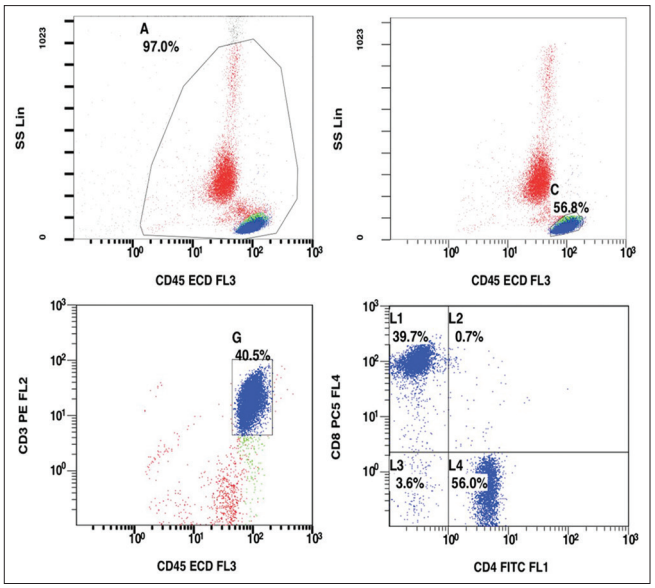

Figure 1: Flow cytometry image showing CD4 and CD8 positive T (CD3 positive) cells

Results

The present study was conducted on children between 1 and 12 years of age. One hundred and twenty subjects were included in the study, out of which 80 had IDA and 40 were healthy age- and sex-matched controls. Table 1 outlines the demographic characteristics of the cases and controls. Clinical features of iron deficiency pallor, atrophic glossitis, and pica were present in 100%, 27.5%, and 20% of the cases (study group), respectively. None of the subjects in the control group had any of these clinical features. Table 2 shows the hematological parameters between cases and controls. As expected, there was a significant difference in the Hb, MCV, and iron parameters between the two groups. On the other hand, serum albumin levels showed no significant difference in the two groups ($P = 0.27$) [Table 2].

The TLC was within normal limits in both the groups. However, it was significantly lower in the IDA group as compared to the control group ($P = 0.03$, standard deviation [SD] – 1948.7 and 1749.8, respectively). Further, total lymphocytes (both absolute and percentage) were significantly raised in iron deficiency ($P = 0.042$, SD – 11.5 and 11.2, respectively). Detailed analysis of lymphocyte subsets showed no significant difference in the percentage of CD3 + T lymphocytes ($P = 0.07$, SD – 9.6 and 8.5, respectively), CD4 + T-helper cells ($P = 0.94$, SD - 10.4 and 7.6), CD8 + cytotoxic T cells ($P = 0.99$, SD – 8.4 and 5.6), and CD4:CD8 ratio ($P = 0.91$, SD – 0.5 and 0.4)

between the two groups. However, CD19 + B lymphocytes were significantly higher in the IDA group as compared to the controls ($P = 0.04$, SD – 4.7 and 4.5) [Table 3].

Spearman correlation test was applied to assess the correlation between the significant outcome variables and hemoglobin and iron parameters (serum iron, serum ferritin, TIBC, and transferrin saturation). No significant correlation was found of Hb and iron parameters with TLC (*r*: 0.18, 0.003, 0.131, 0.18, and 0.040, respectively); with lymphocytes (%) (*r*: 0.05, 0.15, 0.013, 0.015, and 0.145, respectively); and with B lymphocytes (%) (*r*: 0.01, 0.042, 0.131, 0.017, and 0.063, respectively).

Discussion

Anemia, iron deficiency, and infections are complexly interrelated and together account for a large part of the global morbidity and mortality, especially in young children. Iron deficiency has been seen to negatively influence innate immunity and macrophage functions. Some authors have also postulated a negative impact on cell-mediated immunity like thymic atrophy and depression of T-lymphocytes, thus increasing the risk of infections.[6,8] Conversely, infections produce a state of functional iron deficiency by increasing cytokine production and acute phase reactants.

The present study found a significant decrease in TLC in children with iron deficiency as compared to healthy controls ($P = 0.03$). This is similar to the study by Sadeghian *et al*. on premenopausal women.[4] On the other hand, a study done by Ekiz *et al*. in children showed a significant increase in the TLC in subjects with iron deficiency ($P < 0.05$).[5] Despite leukopenia, subjects with IDA presented with relative lymphocytosis ($P = 0.04$). A study done by Aly *et al*. showed similar findings ($P = 0.03$) for total lymphocytes.[8] However, studies done by Das *et al*. and Attia *et al*. found no alteration in the percentage of total lymphocytes in the group of children with IDA.[6,9] Iron is an integral component required for cell cycle enzymes like ribonucleotide-reductase and is involved in DNA synthesis. Thus, it may be inferred that the proliferative phase of lymphocyte activation, which is an iron requiring step, might be affected in patients with IDA. However, the presence of relative lymphocytosis in these patients supports the hypothesis that iron deficiency leads to only a qualitative defect, rather than a quantitative decrease in lymphocytes. This has been validated by some authors.[10]

The relative lymphocytosis in the IDA group was predominantly a result of increase in B lymphocytes as compared to the control group ($P = 0.04$) with no significant difference in the percentages of CD3+ T cells in the two groups. Aly *et al*. also found an inverse correlation between the number of B lymphocytes and iron levels ($P = 0.02$ and $r = 0.27$) suggesting low iron levels might lead to an increase in the number of B lymphocytes, but in their study, IDA subjects had lower CD3+ T cells percentages as well as numbers ($P = 0.005$ and 0.007, respectively) when compared to the control.[8] Similar to our study, Ekiz *et al*. and Keramati *et al*. (in premenopausal women) also found no significant difference

Table 1: Demographic characteristics of cases and controls

	Control group ($n=40$)	Case group ($n=80$)
Age (years), mean±SD (range)	6.2±2.3 (2-10.5)	5.31±3.12 (1-11)
Sex (male/female)	30/10	46/34

SD: Standard deviation

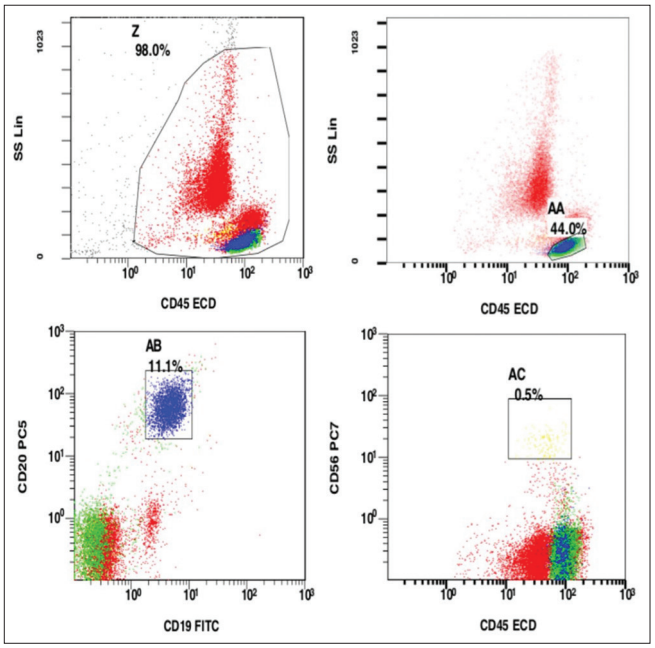

Figure 2: Flow cytometry image showing CD19 CD20 positive B cells and CD56 positive NK cells

Table 2: Iron indices in the iron deficiency anemia and control group

Parameter	Control group ($n=40$)	Case group ($n=80$)	*P*
Age (years)	6.2±2.3	5.31±3.12	0.16
Hb (g/dL)	12.25±0.62	8.09±1.86	<0.0001
Serum ferritin (µg/L)	78.7±18.71	5.27±1.08	<0.0001
Serum iron (µg/dL)	88±19.84	24.63±7.7	<0.0001
Serum TIBC (µg/dL)	276.72±46.65	410.24±64.22	<0.0001
Transferrin saturation (%)	32.44±8.19	6.25±2.15	<0.0001

TIBC: Total iron-binding capacity, Hb: Hemoglobin

Table 3: Immunological parameters in the iron deficiency anemia and control group

Parameter	Control group ($n=40$)	Case group ($n=80$)	*P*
TLC	9261.5±1749.8	8387.7±1948.7	0.031
Total lymphocytes*	36.6±11.2	42.4±11.5	0.042
CD3 + (T cells)*	26.4±8.5	30.7±9.6	0.07
B lymphocytes*	6.6±4.45	8.87±4.73	0.04
CD4+cells**	52.5±7.58	51.9±10.4	0.94
CD8+cells**	38.1±5.6	38.9±8.4	0.99
CD4/CD8 ratio	1.49±0.44	1.48±0.53	0.91

*Out of total leukocytes, **Out of CD3+lymphocytes. TLC: Total leukocyte count

in the percentage of CD3+ T cells between IDA and controls.[5,11] The exact mechanism as to how iron deficiency affects the T and B cell population is not known. Rather than a quantitative effect on numbers, it has been suggested that altered levels of various cytokines and interleukins secreted by effector cells (interleukin [IL]-2, IL-1, IL-6, IL-10, tissue necrosis factor-α, interferon-gamma) might be responsible for cell-mediated immune system impairment in IDA patients.[12]

The study found no significant difference in the percentages of CD4+ helper T cells and CD8+ cytotoxic T cells in the two groups. Iron deficiency has been reported to cause thymic atrophy with decreased T lymphocyte-induced proliferative response. T helper cells (Th1 > Th2) are considered to be more sensitive to iron deficiency than cytotoxic T cells. However, the literature is discordant regarding the quantification of T cell subsets. Similar to the findings of the present study, some studies reported no difference in the percentages of the two subsets in children with IDA.[10,12,13] Aly *et al*. and Das *et al*. reported that the CD4+ T cell population showed significant decrease in the IDA group as compared to the control group ($P = 0.001$ and < 0.001, respectively). However, their finding in the CD8+ population corresponds with ours in that they also found no significant difference in the absolute numbers as well as percentages of CD8+ T cells in the two groups.[6,8] As no alteration was found in the total T cell population as well as the subsets in the present study, it might be inferred that the reduced T cell function documented in the literature is due to functional defects of T cells and/or due to higher proportion of immature T lymphocytes in the IDA patients.

The limitation of the present study was the absence of simultaneous functional assays on lymphocytes which could not be performed due to economical constraints.

Conclusion

Children with IDA had a lower TLC but greater number of lymphocytes mainly contributed by increase in the B lymphocytes as compared to iron-replete children. However, the T cell populations were relatively unaffected by iron deficiency and the impairment in cell-mediated immunity observed by other studies may be functional, rather than quantitative.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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