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Lymphocyte subsets in children with hemophagocytic lymphohistiocytosis (HLH) and its clinical significance

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Abstract. – OBJECTIVE: To study the peripheral blood lymphocyte subsets in children with hemophagocytic lymphohistiocytosis (HLH) in acute period as well as remission period, and compare them with healthy children to investigate the significance of lymphocyte subsets in the diagnosis, treatment, and prognosis in children with HLH.

PATIENTS AND METHODS: From January 2009 to March 2014, 30 HLH patients were enrolled in this study. Among them, 20 were placed in the remission group, while 10 cases were placed in the death group. 6 cases died within 8 weeks due to the illness, and 4 cases died within 9 to 34 weeks. 30 children who were confirmed healthy after physical check-ups in the same period were enrolled in the control group. Peripheral blood was collected from both groups and lymphocyte subsets were studied using flow cytometry.

RESULTS: The ratios of CD3+ T and CD8+ T cells increased in the HLH remission group and the death group, while CD4+, CD4+/CD8+ and CD3-CD16+CD56+NK ratios decreased. Difference detected in the proportion of CD19+ B cells was not statistically significant. By comparing lymphocyte subsets in the HLH acute period and the remission period in HLH patients we discovered that the differences in CD3+, CD8+, CD4+ and CD4+/CD8+, CD19+ B cells ratios were not statistically significant ($p > 0.05$). CD3-CD16+CD56+NK cells ratios in the remission period increased significantly.

CONCLUSIONS: The lymphocyte subsets in children with HLH underwent obvious changes and there was an imbalance in cellular immunity. We believe that dynamic detection of changes may help us to evaluate the prognosis and the effect of the treatment.

Key Words:
Hemophagocytic lymphohistiocytosis, Children, Lymphocyte subsets.

Introduction

Hemophagocytic lymphohistiocytosis (HLH), also known as hemophagocytic syndrome (HPS), is a syndrome caused by the injury due to multiple organ inflammation caused by excessive proliferation and activation of lymphocytes and histiocytes and cytokine storm formation[1-3]. Clinical manifestations include fever, hepatosplenomegaly, reduction in peripheral blood cells, liver dysfunction, coagulation disorders and phagocytosis of hemocyte by histocyte[4,5]. The pathogenesis is not completely understood and the mortality rate is very high[6-9]. Most of researchers believe that immune system abnormalities play a main role in HLH[6]. We, in this study, used flow cytometry to study the peripheral blood lymphocyte subsets in children with HLH in acute period as well as remission period and compared them with healthy children to investigate the significance of changes in lymphocyte subsets in diagnosis, treatment and prognosis of HLH.

Patients and Methods

Patients

From January 2009 to March 2014, 30 cases of newly diagnosed HLH patients who were treated in the Hematology Department of Xuzhou Children's Hospital were enrolled. There were 17 males and 13 females with median age of 1 year (range= 4 months to 10 years). HLH children patients with continuous CR for 40 weeks after treatment were classified in the remission group, while HLH children patients with non-remission or semi-remission were considered as the death group.

20 children were in the remission group, while 10 cases were in the death group. 6 cases died within 8 weeks due to the illness, and 4 cases died within 9 to 34 weeks. 30 children who were confirmed healthy after physical examinations in the same period were enrolled as the control group. There were 19 males and 11 females with the median age of 15 months (range=4 months to

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12 years). Children in the control group did not have any history of acute or chronic diseases, allergies and family hereditary diseases. Differences of gender and age between HLH patients and children in the control group were not statistically significant and were data were proved to be comparable.

Inclusion criteria: (i) patients were between 4 months to 10 years old; (ii) patients were in accordance with HLH-2004 revised standards[10]; (iii) patients did not use hormones, chemotherapy drugs and immunomodulators before a definite diagnosis; (iv) all patients had low blood cell count.

Exclusion criteria: children with tumor related HLH or congenital immunodeficiency were all excluded from this study. HLH - 2004 standards[10] were as follows: (i) Having a fever over 38.5°C; (ii) Presence of splenomegaly; (iii) Low blood cell counts, hemoglobin (Hb) < 90 g/L, blood platelet < 100×10^9/L, absolute neutrophil count (ANC) <1.0×10^9/L; (iv) Fasting serum triglyceride level of ≥3.0 mmol/L, fibrinogen ≤1.5 g/L; (v) Presence of hemophagocytosis in bone marrow, spleen or lymph gland, without the presence of any malignant disease; (vi) Reduction in NK cells activity; (vii) Serum ferritin ≥500 μg/L; (viii) Solubility CD25 (i.e., interleukin-2 receptor) ≥2.4×10^6/L. Those who satisfied five or more of these symptoms were diagnosed as HLH.

This research has obtained the approval of the Medical Ethics Committee of Xuzhou Children's Hospital. All children and parents have participated in this research under the circumstance of knowing the facts, and they have signed the informed consent form.

Sample Collection and Lymphocyte Subsets Detection

Fasting venous blood (1-2 ml) was collected from HLH patients in the acute period, with CR of 40 weeks and children in the control group. We added 50 μl heparin and 20 μl of six color fluorescent monoclonal antibody and mixed them and incubated the sample at room temperature in dark for 15 minutes. BDFACS Lysing was used to dilute hemolysin and then 450 μl was added to each tube. Sample was mixed at room temperature in dark for 10 min. Flow cytometry and lymphocyte subsets analysis software were used for analysis. T lymphocyte surface markers were CD3+, CD4+, CD8+T, B lymphocytes were CD19+, and NK cells were CDl6 + and CD56+.

Statistical Analysis

SPSS 19.0 software was used for statistical analysis. The enumeration data were expressed by percentage and χ^2-test was used for comparison among groups. The measurement data were expressed by mean ± standard deviation. Non-parametric Kruskal Wallis H test was used for comparison among groups and Mann-Whitney U was used for comparison between two groups. $p<0.05$ indicated that the differences were statistically significant.

Results

The ratios of CD3+ T and CD8+ T cells increased in the HLH remission group as well as the death group, and the differences were statistically significant ($p < 0.05$). The CD4+, CD4+/CD8+ and CD3-CD16+CD56+NK ratios decreased, and the differences were statistically significant ($p < 0.05$). Difference detected in the proportion of CD19+ B cells was not statistically significant ($p > 0.05$) (Table I).

With comparing lymphocyte subsets in the HLH acute as well as the remission periods in HLH patients we discovered that the differences of CD3+, CD8+, CD4+ and CD4+/CD8+, CD19+ B cells ratios were not statistically significant ($p > 0.05$).

CD3-CD16+CD56+NK cells ratios in the remission period increased significantly and the difference was statistically significant ($p < 0.05$) (Table II).

Discussion

HLH is a reactive proliferative disease of the mononuclear macrophage system. It has the characteristics of an abnormal proliferation of macrophages and devouring blood cells in histopathology. HLH can be divided into primary HLH and secondary HLH, and the latter is more common. Primary HLH includes familial HLH and the more common HLH caused by primary immunodeficiency syndrome. Secondary HLH usually follows infection, tumor and rheumatic disease[11,12]. Clinical manifestations include fever, liver, spleen and lymph node enlargement, peripheral blood cells reduction, liver dysfunction and blood coagulation disorders. There is no specific treatment for this illness, and the dexamethasone, cyclosporin A (CSA) and etoposide immunochemotherapy are usually used for treatment[10]. Familial HLH usually is treated with hematopoietic

Table I. Comparison of lymphocyte subsets in HLH patients and the control group.

Group Cases	CD3 +	CD8+	CD4+	CD4 + / CD8 +	CD19+	CD16+CD56+
Control 30	67.37±4.33	26.64±3.95	40.05±6.00	1.74±0.54	18.45±3.54	12.31±3.02
Remission 20	71.44±15.02	35.08±19.22	32.88±13.88	1.38±096	16.20±10.90	4.37±2.84
Death 10	80.57±12.57	50.02±26.57	28.53±13.88	1.19±1.06	15.10±11.36	5.39±4.44
H-value	13.683	8.606	15.995	7.857	6.202	45.448
p-value	0.001	0.014	<0.001	0.027	0.096	<0.001

stem cell transplantation[10]. Researchers believe that a defect in cytotoxicity mediated by natural killer cells (NK) and cytotoxic T cells (CTL) leads to overreaction or invalidity of immune system, which results in cytokines storm and multisystem inflammation[6]. If patient suffers from dysfunctional immune regulation and excessive proliferation of immunocompetent cells, then these immunocompetent cells can generate an inflammatory cytokines storm, resulting in HLH. Changes in clinical signs and laboratory parameters can be attributed to the biological role of cytokines[13].

Analysis of lymphocyte subsets is an important indicator for detecting cell immunity and humoral immunity. It can generally reflect immune system's condition and can contribute to diagnosis of some diseases. It also has significance in pathogenesis analysis and the evaluation of prognostic. CD3+ T cells represent the total T cells. CD4+ T cells (T helpers) can contribute to cellular as well as humoral immune systems. The main functions of CD8+ T cells, including CTL and inhibitory T cells, are the specific killing of target cells and secreting inhibitory factors in order to weaken or suppress the immune response.

CD4+/CD8+ are common indicators usually used to judge whether the immune system is functioning properly. Our results revealed that all 30 HLH patients had increased levels of CD3+ T and CD8+ T cells in the acute period, which were significantly higher than those in the control group. CD4+ T cells and CD4+/CD8+ cells ratios were reduced more significantly compared to those in the control group, indicating that HLH patients most probably suffered from cellular immunity dysfunction and excessive proliferation of CD8+ T cells.

Lymphocyte subsets analysis is an efficient way to diagnose HLH. Results obtained from previous studies[14-16] showed that CD8+ T cells increased and CD4+ or CD8+ ratio decreased, which were consistent with the results of the present study. Normal cytotoxic function can limit the excessive activation of the immune system and reduce the development of severe immune pathology[13,14]. Once NK cells and CTLs contact target cells, they begin to carry out a series of cytotoxic activities. This process includes cytotoxic granules synthesis, immunological synapse polarization, release of perforin as well as granule enzymes into the target cells, and inducing apoptosis in target cells. In children with HLH, enzyme dependent cytotoxic function is defective and is not capable of removing the antigen completely. This condition may lead to a continuous antigenic stimulation and uncontrolled as well as excessive production of deadly antigen specific T cells.

Lower levels of perforin can negatively affect the immune response which in turn leads to CD8+ T

Table II. Lymphocyte subsets in the HLH acute period and the remission period in HLH patients.

Group	CD3 +	CD8+	CD4 + / CD8 +	CD19+	CD16+CD56+
Acute	71.44±15.02	35.08±19.22	32.88±13.88	16.20±1090	4.37±2.84
Remission	74.48±14.70	40.06±22.63	31.43±1561	15.80±10.87	6.74±2.38
Z-value	0.352	0.135	1.082	1.001	3.760
p-value	0.725	0.892	0.279	0.372	<0.001

cells activation. CD8+ T cells activation and proliferation can produce large amounts of IFN-γ that stimulates the proliferation and activation of macrophages, and macrophages can release large amounts of inflammatory cytokines, such as TNF-α, IL-1, IL-2, IL-6, IL-10, IL-12, and GM-CSF which creates a cytokine storm, and result in in over inflammatory response and multiple organ damage[17,18].

CD19+ cells regulate the activation and proliferation of B cells and participate in the signal transduction in B cells. CD3-CDl6+CD56+ represents a more mature NK cell and is one of the main components of nonspecific immune system. In this study, all HLH cases showed no significant difference between the ratio of 19+ CD cell in the acute period compared and the control group.

CD3-CD16+CD56+ cells ratio in HLH patients was significantly lower than that of the control group. Therefore, it was speculated that HLH patients had normal humoral immunity, but lower non-specific NK cell immune function. In HLH-2004 program, NK cells activity was added to the diagnostic condition. Results obtained from this study are consistent with the HLH-2004 diagnostic criteria. It has been shown that NK cell activity in HLH patients is significantly lower than that of non HLH patients[19-21]. But in this study, the activity of NK cells could not be detected. Study compared the lymphocyte subsets of 20 patients with HLH in the remission as well as acute periods and showed that only CD3-CD16+CD56+NK cells ratio changed significantly. These results suggested that children with HLH in remission still had cellular immune dysfunction and needed to be monitored closely. Another study[22] reported that the level of IFN-γ in HLH remission children increased slightly compared with that of the control group. This observation was consistent with our results.

Conclusions

We showed that children with HLH in acute period suffered from imbalance in lymphocyte subsets and cellular immunity disorder. We suggest that dynamic detection of changes may help to evaluate the prognosis and the effect of treatment.

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