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Viral Infections: Is it a Possible Etiological Factor of Pediatric Acute Lymphoblastic Leukemia?

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ABSTRACT

Introduction: Pediatric acute lymphoblastic leukemia (ALL) is considered the commonest malignancy affecting children. There is some supportive evidence for an infectious etiology to ALL.

Objective: To evaluate the possible etiological relation between some viral infections (Epstein Barr virus, Cytomegalovirus, Herpes Simplex virus-1, Hepatitis B, C and G viruses) and pediatric ALL.

Patients and Methods: Thirty newly diagnosed ALL patients, with a negative history of blood transfusion, at the National Cancer Institute (NCI), Cairo University were included. Their mean age was 3.6±1.2 years. They were subjected to complete medical evaluation and investigations including complete blood picture and bone marrow aspiration with or without biopsy. Polymerase Chain Reaction (PCR) for Epstein Barr virus (EBV), Cytomegalovirus (CMV), Herpes Simplex virus-1 (HSV-1), Hepatitis B (HBV), C (HCV) and G (HGV) viruses was done for all patients.

Results: PCR results showed seropositivity of 40% for CMV, 36.7% for HSV-1, 10% for each of EBV and HBV, 23.3% for each of HCV and HGV and 6.7% for combined HBV and HCV.

Conclusion: The results of our study are more towards the possibility of viral infections having a role in the etiology of pediatric ALL. Further studies involving larger sample of population and including other viruses are recommended to investigate the viral etiology theory.

Key Words: *Pediatric ALL - Viruses - Infectious etiology.*

INTRODUCTION

Childhood leukemia is a biologically and clinically diverse disease and probably arises via several etiological pathways **[1]**. Only a few cases can be explained by known risk factors, such as ionizing radiation, cancer chemotherapy or Down's syndrome. The etiology of childhood leukemia remains uncertain, but recent research has provided new clues. Both genetic susceptibility and environmental exposures are likely to be involved.

It is possible that both prenatal and postnatal environmental exposures may play a crucial role in triggering the onset of leukemia. The process leading to the onset of childhood leukemia is likely to involve at least two events **[2,3]**. Whilst the first event may be either germ line (although this appears to be rare in childhood leukemia) or somatic because of endogenous or environmental factors, the final 'critical' event may always involve an environmental factor. Both events would lead to cellular genetic changes and/or the proliferation of premalignant clones. Infections have been considered a prime candidate for such environmental etiological agents that promote the onset of leukemia. Epidemiological evidence suggests that infection is involved either by stimulating an inappropriate immune response or in the form of a classical transforming agent **[4]**. Several observations contribute to the theory that a transmissible agent is potentially involved in the oncogenic process of childhood leukemia. First, the peak incidence of childhood leukemia and that of common childhood infections both occur among children 2-5 years of age, the age group least likely to possess sophisticated immune systems **[5]**. Second, a viral etiology has been shown for some animal and human cancers (e.g. EBV for Burkitt lymphoma) **[1]**. Third, evidence exists of an apparent seasonal variation in the onset of childhood leukemia. Statistically significant seasonal variation for ALL with a peak in the

summer **[6]**, in the autumn-winter **[7]** and in the early spring among 1- to 6-year-old children **[8]** have been described.

Several viruses have been incriminated in the causation of ALL in childhood. Viral particles have been isolated in cultures of mononuclear cells from patients with ALL **[9]**.

The aim of our study is to evaluate the possible etiological relation between some viral infections (EBV, CMV, HSV-1, HBV, HCV and HGV) and pediatric ALL.

PATIENTS AND METHODS

Patients:

Thirty patients with ALL diagnosed and managed at NCI, Cairo University were randomly selected to participate in this study. All patients were newly diagnosed by means of complete blood picture and bone marrow aspiration with or without biopsy. They all had a negative history of blood transfusion. Patients with ALL associated with other possible known etiology e.g. Down syndrome were excluded.

Detection of active viral infection was done using Polymerase Chain Reaction (PCR) for EBV, CMV, HSV-1, HBV, HCV and HGV.

Methods:

Clinical evaluation of the patients included full history taking and thorough physical examination. Laboratory investigations included complete blood picture, bone marrow aspiration with or without biopsy and PCR for EBV, CMV, HSV-1, HBV, HCV and HGV.

Collection of serum samples:

Five milliliters of peripheral venous blood were collected from patients by venipuncture for PCR assessment. Samples were centrifuged to separate the sera. The latter were kept at a temperature of –80ºC till PCR assessment.

Molecular biology methods:

I- *RNA Extraction for detection of HCV and HGV-mRNAs:*

Total RNA was extracted from all sera using SV total RNA extraction kit provided from Promega Corporation, Madison, WI, USA. The amount of RNA was quantitated by reading the Optical Density (OD) at wave length of 260nm by using spectrophotometer.

A- *cDNA synthesis and PCR of HCV:*

The RT-PCR method here is the one step method. The kit was provided from Qiagen. The RT-PCR amplification protocol was formed of: Qiagen one step RT-PCR buffer (5x), dNTPs (10µM), primer (1CH, 10µM), primer (2CH, 10µM), Qiagen one RT-PCR enzyme mix and template RNA in a total volume of 50µL. The oligonucleotide primer sequences used for amplification of HCV were illustrated in Table (A)

The PCR cycling condition was as follows: one cycle of 50ºC for 30min, one cycle of 95ºC for 15min followed by 40 cycles of 95ºC for 1min, 45ºC for 1min and 72ºC for 1min. Then extension for 10min at 72ºC. To increase the sensitivity of the RT-PCR assay of HCV, 3µL of the first amplified product was subjected to nested PCR. The sequences of the nested oligonucleotide primers (4CH & 1TS) were illustrated in Table (A).

B- *cDNA synthesis and PCR of HGV:*

The RNA was reverse transcribed by using random primer, first strand buffer, dNTPs, RNase inhibitor and MMLV. The total volume of 50µL was introduced to PCR apparatus for one hour at 37ºC followed by 5min at 95ºC. The sequence of the primers used was illustrated in Table (A). The PCR mixture was performed in a total volume of 100µL reaction volume containing 5µL of the cDNA, buffer 2 (50mM KCl, 0.01% gelatin and 10mM Tris HCl, pH 8.3), $MgCl_2$ (2mM), dNTPs (100mM each), primer (101-sense), primer (285-antisense) and Taq polymerase (5u/µL). The PCR cycling condition was: 95ºC for 5min followed by 40 cycles of 94ºC for 1min, 55ºC for 1min and 72ºC for 1min.

II- *DNA Extraction for detection of HBV, HSV, CMV and EBV:*

DNA was extracted from plasma samples using the QIAamp® DNA minikit (Qiagen, USA) following the manufacturer's instructions. The extracted DNA was quantified and checked for purity using a spectrophotometer.

A- *PCR of HBV:*

The PCR mixture was performed in a total volume of 100µL reaction volume containing DNA, 10X buffer, $MgCl_2$, dNTPs, primer (T801-sense), primer (T935-antisense) and Taq polymerase (5u/µL). The primer sequences were

illustrated in Table (A). The PCR cycling condition was: 95ºC for 10min followed by 55 cycles of 94ºC for 20sec, 60ºC for 20sec and 72ºC for 30sec. The PCR products were detected by electrophoresis on 2% agarose gel stained with ethidium bromide and photographed under UV.

B- *PCR for CMV and HSV:*

The PCR mixture was performed in a total volume of 50µL reaction volume containing DNA, 10X buffer, $MgCl_2$, dNTPs, specific primers and Taq polymerase (5u/µL). The primer sequences were illustrated in Table (A). The PCR cycling condition was: 95ºC for 5min followed by 35 cycles of 94ºC for 60sec, 60ºC for 60sec for CMV and 57 for HSV and 72ºC for 60sec.

C- *PCR for EBV:*

A nested-PCR method was used to detect viral DNA of EBV-1 and EBV-2. The primers had demonstrated high specificity and no cross-reactivity with the human genome. The sequences of EBV outer and inner primers were illustrated in Table (A). The first round PCR reaction was carried out in a volume of 40µl total mixture that included 5µl of the template, 1xPCR buffer (pH 8.3) and 1.25U Taq DNA polymerase, 0.2mmol/L of each dNTP, 1.5mmol/L $MgCl_2$, and 50pmol/L of the EBV outer primers. PCR amplification included an initial denaturation step at 94°C for 5min followed by 30 cycles of denaturation steps at 94°C for 1min, primer annealing at 60°C for 1min and an extension step at 72°C for 1min, and then a final extension step at 72°C for 7min. In a new tube, a second round of amplification was performed using 2µl of the first round PCR product, 50pmol of the inner primers for EBV, and PCR buffer, Taq DNA polymerase, dNTP and $MgCl_2$ in concentrations described above. The program for the second PCR was 35 cycles of denaturation step at 94°C for 1min, an annealing step at 55°C for 1min and an extension step at 72°C for 1.5min, and then a final extension step at 72°C for 7min.

III- *Agarose gel electrophoresis:*

Ten µL of the PCR product was separated electrophoretically in a 1.5% agarose gel stained with ethidium bromide and observed under UV light. The expected length of the PCR product was as illustrated in Table (A). Negative and positive controls were included in every run. In parallel, a DNA marker was separated.

Data management and statistical analysis:

The data was coded and entered on an IBM compatible personal computer using the statistical package SPSS ver. 9.0. The mean ± standard deviations were calculated for the numerical data.

Table (A).

	Oligonucleotide sequences	Product size
1CH	5'GGTGCACGGTCTACGAG ACCTC3'	
2CH	5'AACTACTGTCTTCACGC AGAA3'	289bp
4CH	5'ATGGCGTTAGTATGAGT G3'	
1TS	5'GCGACCCAACACTACTC GGCT3'	187bp
101-sense	5'CGGCCAAAAGGTGGTG GATG3'	
285-antisense	5'CGACGAGCCTGATGTTG GG3'	185bp
T801-sense	5'GCTACGTCACTAACCAC GTG3'	
T935-antisense	5'CTACGGTGTGTAAACTC ACC3'	200bp
F (CMV)	5'TTGCAGGCCACGAACA ACGT3'	
R (CMV)	5'GTCTACGGATTGCTGAC GCT3'	305bp
F (EBV) outer	5'-AGG GAT GCC TGG ACA CAA GA-3'	
R (EBV) outer	5'-TGG TGC TGC TGG TGG TGG CAA-3'	432bp
F (EBV) inner	5'-TCT TGA TAG GGA TCC GCT AGG ATA-3'	
R (EBV) inner	5'-ACC GTG GTT CTG GAC TAT CTG GAT	160bp
F (HSV)	C-3'	
R (HSV)	5'-CGGAATTCCGTCATCT CA CGGGGACAC-3'	324bp
	5'-CGGGATCCCGACGGT ATC GTCGTAAA-3'	

RESULTS

Among the thirty studied patients, 16 were males (53%) and 14 were females (47%) with male to female ratio of 1.14:1. Their mean age was 3.6±1.2 years. The PCR results for the studied patients are shown in Table (1) and Fig. (1).

Pallor was the commonest sign observed in all patients. Rash was evident in 18 (60%) of our patients. Cervical lymphadenopathy was present in 26 patients (86.7%), hepatomegaly in 22 patients (73.3%) and splenomegaly in 23 patients (76.7%).

The mean hemoglobin of studied patients was 7.42±1.95gm/L (ranging from 2.6 to 11gm/L). The total leucocytic count was increased in 24 patients (80%) with a mean of 26.17±16254/cmm (with a range of 1050 to 71000/cmm). Their mean platelet count was 42866±34129/cmm. Diagnostic blast cells in both peripheral blood and bone marrow were evident in all patients.

Table (1): PCR results among studied patients (n=30).

PCR positive	No.	Percentage
CMV	12	40
HSV-1	11	36.7
EBV	3	10
HBV	3	10
HCV	7	23.3
HGV	7	23.3
Mixed HBV & HCV	2	6.7

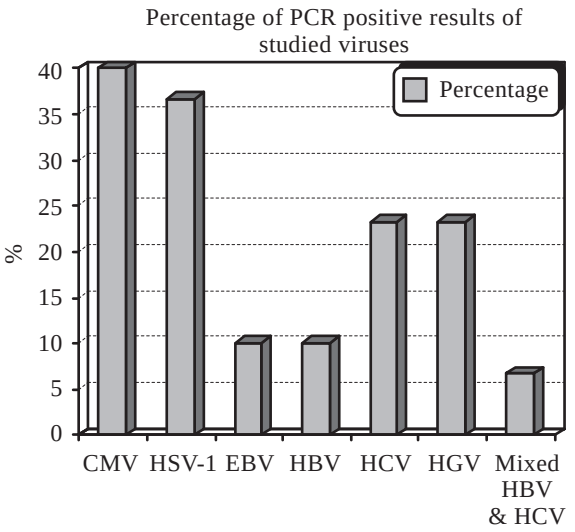

Fig. (1): Percentage of PCR positive results of studied viruses.

DISCUSSION

Leukemia is the commonest form of cancer in children accounting for around a third of all childhood cancer **[10]**. ALL accounts for approximately three-quarters of all pediatric leukemia diagnoses **[11]**.

There are three current hypotheses concerning infectious mechanisms in the etiology of childhood leukemia: exposure in utero or around the time of birth, delayed exposure beyond the first year of life to common infections and unusual population mixing. No specific virus has been definitively linked with childhood leukemia and there is no evidence to date of viral genomic inclusions within leukemic cells **[12]**. This study was carried out to investigate the possible etiological relation between some viral infections (EBV, CMV, HSV-1, HBV, HCV and HGV) and pediatric ALL.

This study included 30 (16 males and 14 females) newly diagnosed ALL patients with a mean age of 3.6±1.2 years.

The seropositivity of screened viruses in our study carries the possibility of viral etiology of ALL, which agrees with Greaves' hypothesis **[3]**, who suggested that the most common form of childhood ALL, common ALL, which is responsible for the age peak, may be due to two separate genetic events **[3,13]**. The first event is thought to be a spontaneous mutation in a B cell precursor and occurs in utero. This transformed B cell precursor clone will proliferate when exposed to a later antigenic challenge. The second stage is influenced by external agents, which results in an expansion of the transformed B cell clone into clinically overt ALL. Furthermore, delay in the normal pattern of exposure of the immune system to infection might lead to an increase of the number of susceptible preleukemic cells and thus the chance of the second critical mutation occurring, leading to overt leukemia **[3]**. It was also suggested that no specific infectious agent is involved but reduced antigenic challenge in infancy can lead to increased proliferation of a preleukemic clone when a later infection occurs.

The evidence of viral infection among our studied patients also goes with another hypothesis which suggests that common ALL may be a rare response to an unidentified mild or sub

clinical infection, the transmission of which is facilitated when large numbers of people come together, particularly from a variety of origins **[14,15]**. Another model, called the aberrant response model, suggested that a substantial proportion of childhood ALL cases arise as a rare host response to certain patterns of exposure to common infectious agents **[16]**. A specific, but yet unknown transmissible agent is causally associated with childhood ALL. For children diagnosed in the childhood peak, primary infection may occur shortly before diagnosis, while for other ages, attention has focused on gestational/neonatal exposure leading to persistent infection. There is also evidence that population density is correlated with childhood ALL risk, which may be indicative of a viral involvement **[17]**. There is some supportive evidence for an infectious etiology provided by the findings of space-time clustering and seasonal variation **[12]**. Genetically determined individual response to infection may be critical in the proliferation of preleukemic clones as evidenced by the human leukocyte antigen class II polymorphic variant association with precursor B-cell and T-cell ALL **[18]**. Similarly, three studies showed a statistically significant increased risk associated with childhood exposure to some infections **[19,20,21]**. However, some studies showed a statistically significant protective effect **[22,23,24]**. One study showed a statistically significant protective effect of infection with roseola (fever plus rash) against ALL when exposure occurred during the first year of life, but a statistically significant increased risk of developing ALL for those with tonsillitis 3-12 months before diagnosis **[21]**. Other authors supported that early common infections may play a protective role in the etiology of childhood leukemia, although this effect was not more marked for ALL **[25]**.

For infection-related hypothesis, the agent must be common in childhood, should also infect adults and should be able to establish persistent infection. There have been limited studies to date trying to define the organism(s) to which abnormal immune responses might lead to the conversion of a preleukemic clone into overt leukemia **[12]**. Several viruses were blamed in the causation of ALL including cytomegalovirus **[26]**, herpes virus **[23]** and varicella zoster **[27]**.

Our results showed CMV seropositivity of 40% among all patients. This agrees with the statement that CMV is one of the viruses incriminated in the causation of childhood ALL by arresting maturation of lymphocytes and megakaryocytes in bone marrow, thus causing proliferation of their immature forms due to dysregulation of cycline E gene expression in human infected cells **[28]**. In another study, it was found that 75% of B-ALL samples of malignant cells at diagnosis were positive for CMV using real time quantitative PCR, and CMV loads were significantly higher in B-ALL samples than in multiple myeloma, B-chronic lymphocytic leukemia or acute myeloid leukemia samples **[29]**. In 2003, a case report was described with CMV infection inducing thrombocytopenia and B-lymphocytes progenitor-precursor cells **[30]**. On the other hand, some authors investigated the presence of prenatal CMV infection in children who later developed ALL and their results showed that prenatal CMV infection does not seem to be associated with later development of childhood ALL **[31]**.

Among our patients, HSV-1 seropositivity was 36.7%. In a study on 68 newly diagnosed pediatric patients with ALL, significantly high percentage of HSV-1 and 2 IgM or reactivated infection was found among leukemic children, 17/68 (25%) compared with normal control 0% and the prevalence of HSV-1 and 2 IgG increased from 18/33 (54%) in children <5 years to 11/13 (77%) in children >10 years, and reactivation of HSV-1 and 2 increased with increasing age from 1/33 (3%) in children <5 years to 4/13 (30%) in children >10 year **[32]**. On the other hand, screening for herpes virus genomes in common ALL showed that, in spite of being present at low levels, detection rates and levels were similar in leukemic and control panels; so it was concluded that a herpes virus is unlikely to be etiologically involved as a transforming agent in common ALL **[33]**. Otherwise, the literature was deficient as regard the etiological relation of HSV-1 and ALL.

In our study, EBV seropositivity was found in 10% of all patients. This agrees with a study that showed that children under 6 years with acute leukemia were more likely to be EBV seropositive than age-matched controls **[34]**. However, another study found that, for children aged 0-4 years, leukemia was inversely associated with EBV seropositivity **[20]**.

The relation of EBV and infectious mononucleosis, pharyngeal carcinoma and Burkitt's lymphoma is well known **[35]**. The higher immunologic response against EBV suggests that such agent could play a role in the onset of ALL in children, if not as a direct etiological factor. Some studies have reported a significantly increased risk for childhood leukemia associated with maternal infection during pregnancy, specifically, EBV infection **[36]**. ALL, a rare complication of viral infection, was reported to follow EBV infection, where infection of T-cells at an early stage of differentiation may lead to failure of normal T-cell repertoire development with subsequent autoimmunity or malignancy **[37]**.

HCV infection is a common health problem in Egypt with a high prevalence rate. A seropositivity of 12% in normal children was reported and it was stated that a relatively higher prevalence of HCV antibody seropositivity was found in healthy Egyptian children compared to reports from other countries **[38]**. Another study showed that the prevalence of HCV antibodies increased from 2.7% in those <20 years of age to more than 40% in males aged 40-54 years in rural areas of Egypt **[39]**. HGV is a single stranded RNA virus which belongs to the flavivirus family. Its genome structure resembles that of HCV, but its transmission is largely independent of HBV and HCV. No association was found between HGV viremia and hepatitis, or with co-infection with either HBV or HCV **[40]**. A relatively high prevalence of GBV-C/HGV-RNA was reported among different Egyptian groups compared to international figures **[41]**. Moreover, despite of compulsory hepatitis B vaccination in Egypt, one study showed that HBV infection occurred in 6.8% of vaccinated children **[42]**.

High incidence of HBV and HCV infection was noticed amongst patients with lymphoproliferative disorders with increased carrier rate **[43]**. HGV shares genetic and biological features with HCV, thus it might also be involved in lymphomagenesis **[44]**. Hypoplastic anemia, a precursor of lymphoblastic leukemia, was reported as sequel of Non-A, Non-B viral hepatitis infection **[45]**. Our results showed seropositivity of 23.3% for each of HCV and HGV. We also found that 10% and 6.7% of our patients had HBV and combined HBV and HCV respectively. HCV was reported in 7.6% of ALL patients and it was suggested that HCV infection may be associated, not only with B-Non Hodgkin lymphoma, but also with some other lymphoid and myeloid malignancies **[46]**.

All our studied patients were diagnosed and managed at the NCI, which is a governmental free of charge referral center for most rural and urban areas with low socioeconomic standard in Egypt. Thus, the patients' socioeconomic standard varied from low to middle class. Some authors indicated that the risk of common ALL increases by higher socioeconomic status, isolation, and other community characteristics suggestive of abnormal patterns of infection during infancy **[1]**. Children in developed countries are up to 4 times more likely to get leukemia than those living in developing countries and the risks are higher for rich children **[47]**. The marked childhood peak in resource-rich countries and the increased incidence of childhood peak in ALL ages 2-6 years predominantly with precursor B cell ALL is supportive of the concept that reduced early infection may play a role **[12]**. It was suggested that socioeconomic factors, associated with community characteristics rather than individual lifestyle, are related to the risk of childhood leukemia and that these factors act early in life **[48]**.

Our results showed that pallor was the commonest sign, being evident in all patients. This agrees with most studies that report pallor as a basic sign of ALL **[49]**.

The role of viral infections as an etiological factor for childhood ALL is still debatable, but the results of our study are more towards this possibility. Further studies involving larger sample of population and including other viruses are recommended to investigate the viral etiology theory. Vaccinations, if available, as means of prophylaxis against these infections as well as close follow up of patients for evaluation of the fate of these viral infections are recommended.

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