Seroprevalence of herpes simplex virus types 1 and 2, Epstein–Barr virus, and cytomegalovirus in children with acute lymphoblastic leukemia in Egypt

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ABSTRACT

Objectives: Viral infection, especially caused by herpes viruses, is now recognized as an important cause of morbidity and mortality in immunocompromised cancer patients. This study aimed at studying seroprevalence of 3 herpes viruses Herpes simplex virus types 1 and 2 (HSV 1 and 2), Epstein-Barr virus (EBV), and cytomegalovirus (CMV) in children with acute lymphoblastic leukemia (ALL).

Methods: We conducted this study on 68 newly diagnosed pediatric patients with ALL presented to the Pediatric Oncology Service of National Cancer Institute, Cairo University, Egypt from November 2001 to June 2003. We used enzyme-linked immunosorbent assay in detecting HSV 1 and 2, CMV, EBV antibodies of both types immunoglobulin (Ig) M and IgG detection of DNA for both CMV and EBV by polymerase chain reaction was carried out.

Results: High seroprevalence of HSV-1 and 2, CMV and EBV IgG antibodies in both leukemic children and their control was observed (69%, 100%, 83%) and (80%, 100%, 95%). Significantly higher percentage of HSV-1 and 2 IgM or reactivated infection was found among leukemic children 17/68 (25%) compared with normal control 0%. Analysis showed that 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. This was in contrast to seroprevalence of CMV and EBV IgG which were 100% and 83% in children <5 years. No difference in seroprevalence was found among both gender, and no difference was found in leukemic patients with granulocytopenia.

Conclusion: The data show a higher exposure to HSV-1 and 2 both primary infections and reactivation among ALL children. Therefore, acyclovir prophylaxis could be highly effective for seropositive leukemic patients who are undergoing induction chemotherapy.

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Since the prognosis and overall survival of children with cancer have dramatically improved during the past 30 years, problems remain related to infections, mainly during leukopenic periods. Viral infections are a frequent cause of severe disease in children with hematological malignancies, cancer

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or patients undergoing allogeneic hematopoietic stem cell transplantation. Due to the underlying disease itself or antineoplastic therapy leading to granulocytopenia, lymphopenia, disruption of natural skin, mucosal barriers, or reduced humoral immune function.[1] Recognition of viral infections, monitoring, prophylaxis and treatment are aimed at reducing the number of infected patients, mitigating the cause of the disease and limiting deaths directly linked with infections in pediatric cancer patients.[2] Viral infection, especially that caused by the herpes group of viruses, is now recognized as an important cause of morbidity and mortality for patients with significant cellular immunosuppression.[3]

Herpes viruses constitute a group of large envelope DNA containing viruses. The members of this group have the ability to establish latent infections; reactivation could take place during periods of immunosuppression.[4] It is reported that in patients with acute leukemia two thirds of seropositive patients could develop reactivation of herpes simplex virus (HSV) infection during remission induction therapy.[3] The risk of cytomegalovirus (CMV) in acute leukemia patients was recognized almost 30 years ago,[5] Epstein-Barr virus (EBV) infection could be associated with lymphoproliferative disorders in patients with acute lymphoblastic leukemia (ALL).[6] The aim of the present study is to investigate the seroprevalence of 3 herpes viruses; namely, HSV, CMV, and EBV and to explore the possible reactivation of these viruses, to prevent further deterioration and severe complications associated with reactivation of these viruses.

Methods. The study was composed of 68 newly diagnosed pediatric patients with ALL presented to the Pediatric Oncology Service of National Cancer Institute, Cairo University, Egypt from November 2001 to June 2003. Twenty siblings were also included as normal controls. The study was conducted on 68 newly diagnosed pediatric children ≤16 years. Diagnosed as ALL from 0-2 weeks. Acute lymphoblastic leukemia patients >16 years were not included, ALL patients who started chemotherapy were not included. Examinations performed in all patients included a full history, clinical examination, a battery of investigations for diagnostic purpose, and liver and kidney functions for all leukemic children. These test includes urea, creatinine, aspartate transaminase, alanine aminotransferase, albumin and prothrombin time.

Laboratory methods. Five ml venous blood was withdrawn from each subject under study and processed as follow: sera were separated from 7 ml of coagulated blood and kept at -20^{O}C in aliquots until examined.

Anti-HSV-1 and 2, IgM and IgG AB were carried out using enzyme linked immunosorbent assay (ELISA) kit (Diagnostic Systems Laboratories, Inc, Texas, USA). The antigen is composed of purified and inactivated HSV-1 and 2; the tests were carried out according to the manufacturer's instructions.

Anti-EBV viral capsid antigen (VCA), IgM and IgG AB was carried out using ELISA kit (Diagnostic Systems Laboratories, Inc. Texas, USA). The antigen is composed of purified and inactivated Epstein Barr VCA. The presence of IgM against VCA indicates a current infection, whereas the presence of IgG against VCA indicates prior infection.

Anti-CMV, IgM and IgG AB was carried out using ELISA kit (Diagnostic Systems Laboratories, Inc. Texas, USA). The antigen is composed of purified and inactivated CMV. The presence of specific IgM antibodies indicates the primary infection, whereas presence of specific IgG antibodies indicates the immune status of patients, the tests were completed according to the manufacturer's instructions. Quantitative results were determined in the form of arbitrary unit (AU)

$$AU = \frac{\text{Optical density of sample}}{\text{Optical density of cut-off}} = \text{index of positivity}$$

The sample is positive if the IgG concentration is >10 IU/ml, negative if the concentration is <10 IU/ml. Reactivation of the infection was considered if the concentration of IgG is 2-fold or ≥10 Iu/ml.

Molecular detection for EBV and CMV DNA in serum. Extraction of DNA from serum of each individual was carried out using silica gel method. The nucleic acid is finally eluted in Tris Ethylene diamine tetraacetic acid buffer and can be amplified directly.

Primer pairs were constructed to amplify the N terminal region of Epstein Barr nuclear antigen (EBNA)-1 gene by outer primer, nucleotide position 109151-109759. The amplification was carried out using 10 μl of extracted DNA in a final volume of 50 μl of 2.5U/100 μl of Amplitaq (Perkin-Elmer Cetus, Norwalk, CT) containing 20 pmol of each EBNA-1 outer primer, together with 10 x buffer, 10 mmol/L nucleoside triphosphate (dNTPs) using the following thermal cycle program (Perkin-Elmer Cetus 9700): 94^{O}C for 5 minutes, 94^{O}C for 30 sec, 58^{O}C for 30 sec and 72^{O}C for 40 sec. This was repeated for 25 cycles with a final extension at 72^{O}C for 10 min. In the second amplification [(nested polymerase chain reaction (PCR)] was performed under the same conditions, except that 5 μl of the first-round of PCR product and EBNA-1 inner primers were used, nucleotide position

109266-109573. As a negative control, blank reagent that contained 10 μl of H_2O was used instead of 10 μl of nucleic acid. A 10 μl of positive control sample diluted in a ratio of 1:10 (DNA extracted from EBV-positive cell line, B95-8) were processed in parallel with every nested PCR run.

The products of nested PCRs were analyzed by electrophoresis on 1.5% agarose, visualized after ethidium bromide staining, and photographed, the expected EBV band was at the level of 308 bp fragment.

Polymerase chain reaction for amplification of CMV DNA. Oligonucleotide primers chosen are localized in exon 1 of the major CMV immediate early gene, nucleotide position the amplification was carried out using 10 μl of extracted DNA in a final volume of 50 μl of 2.5U/100 μl of Amplitaq (Perkin-Elmer Cetus, Norwalk,CT) containing 10 x PCR buffer, 1.5 mM $MgCl_2$, 20 pmol of each primer, 10 mmol/L dNTPs using the following thermal cycle program (Perkin-Elmer Cetus 9700): 94°C for 5 min, 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. This was repeated for 30 cycles with a final extension at 72°C for 10 min. As a negative control, blank reagent that contained 10 μl of H_2O was used instead of 10 μl of nucleic acid. A 10 μl of positive control sample diluted in 1:10 [DNA extracted from CMV-positive cell line, MRC-5 (cell line from human lung fibroblast)] were processed in parallel with every PCR run. The products of PCRs were analyzed by electrophoresis on 1.5% agarose, visualized after ethidium bromide staining, and photographed; the expected CMV band was at the level of 290 bp fragment .

Statistical analysis. Statistical package for social sciences version 13.0 was used for analyzing the data. Chi-square test was used for comparison of independent proportions. Non-parametric t-test was used for 2 independent groups. *P* value is significance at <0.05 level.

Results. Age and gender distribution of the 88 individuals included in the study is shown in **Table 1**. The median age of the 68 pediatric acute leukemias was 6 years, and that of the 20 apparently healthy siblings was 14 years. Preponderance of males was observed among acute leukemia patients with a male to female ratio of 1.8:1 (**Table 1**). The total exposure of anti HSV-1 and 2, IgG and IgM antibodies was in both acute leukemia cases (73%) and normal siblings (80%) (p=0.11). However, when comparing the seroprevalence of HSV-1 and 2 primary infection (HSV IgM) or reactivated infection (high titer of HSV IgG), leukemic children had significantly higher positivity (17/68) (25%) compared with 0% in normal control group p<0.005 (**Table 2**). As regard with seroprevalence of CMV antibody, all 68 leukemic patients and 20 normal control (100%) had anti CMV IgG antibody, and all patients (68/68) and 19/20 of normal control group were negatives for CMV IgM antibody (**Table 2**). For seroprevalence of EBV antibody, there was also no difference in the seroprevalence of EBV IgG between both leukemic (83%) and control group (95%). However, seroprevalence of EBV IgM was higher in the normal control group than in the leukemic group 3/20 (15%) versus 3/68 (4%) but this difference was not significant (p=0.12) (**Table 2**). The presence of EBV and CMV genomes were tested by qualitative PCR assay in serum of both leukemic and normal control groups. Of 68 leukemic children, 2 (3%) were found to be positive for CMV DNA, and 5 (6%) were found to be positive for EBV DNA in serum by PCR. Of the 20 normal control individuals, no one showed positive CMV DNA in serum but EBV DNA could be detected in the serum of 2/20 (10%) by PCR assay (**Figures 1 & 2**).

Analysis of 3 herpes viruses Ab titer among different age group was performed, 3 age groups were studied, those with (<5 years), 5-10 years and >10 years. Leukemic children at the preschool age (<5 years) showed a lower HSV IgG (18/33 [54.5%]) compared with older age group (11/13 [77%]). However, 100% prevalence of CMV IgG showed among children <5 years, and no difference in prevalence of EBV IgG in young and old children (**Table 3**). Furthermore, leukemic children who showed reactivation of HSV (high titer of HSV IgG) was higher in old age group than in the young age group (p=0.006). Almost the same was found among normal control group. In leukemic patients, no significant difference was shown in seroprevalence of HSV-1 and 2, CMV, and EBV IgM and IgG in males versus females (**Table 3**) (p=0.3). In normal control females (83%, 25%, 100%) showed higher seroprevalence although not significant of HSV-1 and 2 IgG, EBV IgM and IgG than males (75%, 0%, 87.5%) (p=0.25). Leukemic patients with neutropenia showed no difference in seroprevalence of 3 herpes viruses than patients without neutropenia (p=0.19) (**Table 3**). Out of 68 leukemic children, 29 (42%) showed abnormal levels of alanine aminotransferase (ALT) value and a high percentage of abnormal ALT level was found in patients who had positive HSV-IgM (6/8 [75%]) compared with EBV IgM (2/3 [66.7%]) while patients with positive CMV IgG (28/68 [41%]) was found to have a high ALT level.

Discussion. In 2000, malignancy of the lymphatic and hematopoietic tissues constitutes 7%

Table 1 - Distribution of age and gender in all subject groups.

Group	No. of cases	Age (years)		Gender		
		Range	Median	Male	Female	M:F
Acute leukemias	68	0.5 - 14	6	44	24	1.8:1
Normal controls	20	1 - 16	14	8	12	0.6:1
Total	**88**	**0.5 - 16**	**8**	**52**	**36**	**1.4:1**

Table 2 - Seroprevalence of herpes viruses antibodies (HSV, CMV, EBV) in all subject groups.

Group	No. of cases	HSV 1 and 2			CMV		EBV	
		IgM N (%)	IgG N (%)	High IgG N (%)	IgM N (%)	IgG N (%)	IgM N (%)	IgG N (%)
Acute leukemia group	68	8 (11.7)	47 (69)	9 (19)	0 (0)	68 (100)	3 (4.4)	57 (83)
Normal control group	20	0 (0)	16 (80)	0 (0)	0 (0)	20 (100)	3 (15)	19 (95)

HSV - Herpes simplex virus, CMV - cytomegalovirus, EBV - Epstein-Barr virus, Ig - immunoglobulin

Table 3 - Risk factors related to prevalence of herpes viruses in leukemic children.

Factor	HSV 1 and 2 +ve			CMV +ve	EBV +ve	
	IgM N = 8 N (%)	IgG N = 47 N (%)	High IgG N = 9 N (%)	IgG N = 68 N (%)	IgM N = 3 N (%)	IgG N = 57 N (%)
Age						
<5 years (n=33)	6 (18)	18 (54.5)	1 (3)	33 (100)	0 (0)	25 (75.7)
5 - 10 years (n=22)	1 (4.5)	18 (81)	4 (18)	22 (100)	2 (9)	20 (91)
>10 years (n=13)	1 (7.6)	10 (77)	4 (30.7)	13 (100)	1 (7.6)	12 (92)
Gender						
Males (n=44)	6 (13.6)	31 (70)	7 (16)	44 (100)	2 (4.5)	35 (79.5)
Females (n=24)	2 (8)	16 (66.6)	2 (8)	24 (100)	1 (4.2)	22 (91.7)
TLC						
<4 x 10^3 (n=12)	2 (16.7)	8 (66.7)	1 (8)	12 (100)	1 (8)	11 (91.7)
>4 x 10^3 (n=56)	6 (10.7)	39 (69.6)	8 (14)	56 (100)	2 (3.5)	46 (82)

HSV - Herpes simplex virus, CMV - cytomegalovirus, EBV - Epstein-Barr virus, Ig - immunoglobulin, TLC - total leukocytic count

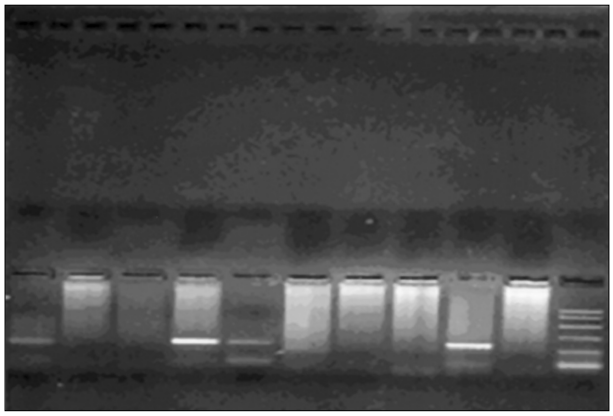

Figure 1 - Polymerase chain reaction for cytomegalovirus DNA showing positive (lanes 1, 4, 5, 9) (lane 11) 100 bp ladder. Positive signals are 290 bp.

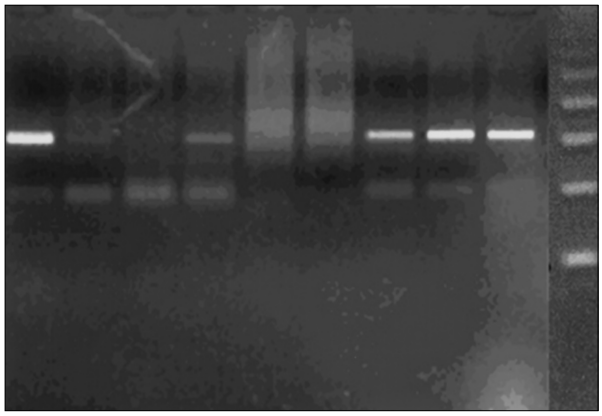

Figure 2 - Polymerase chain reaction for Epstein-Barr virus DNA showing positive (lanes 1, 2, 4, 7, 8, 9).

of the total malignancies presented at the National Cancer Institute, Cairo University as reported by El-Boulkainy.[7] However, treatment of ALL is one of the true successes of modern oncology. The incidence of ALL is higher among boys than girls. There appear to be geographic differences in the frequency and age distribution of ALL. In the present study, 68 cases of Egyptian childhood acute leukemics showing a median age of 6 years was similar to that reported from the USA.[8] Viruses from the herpes group [HSV, varicella zoster virus (VZV), EBV, CMV] remain a significant cause of morbidity and mortality in iatrogenically immunosuppressed individuals despite the considerable progress achieved in recent years by the use of antiviral drugs. The seroprevalence of HSV-1 infection in the general population is in excess of 70%, and the prevalence of HSV-2 varies with socioeconomic status, occupation, and age, but is approximately 25%. Herpes simplex virus infection that occurs in a patient with a hematologic malignancy may be more frequent, severe, and prolonged than in non-immunosuppressed individuals. This is depending upon the specific underlying illness, the disease activity, and the temporal relationship to initiation of cytotoxic immunosuppressive therapy.[4] Furthermore, visceral involvement of liver, lungs, adrenal glands, central nervous system, and skin has been reported in immunocompromised patients. The presence of circulating anti-HSV antibodies is an indication of potential risk of recurrent infection. In HSV seropositive patients with acute leukemia who were undergoing induction chemotherapy with cytarabine and daunorubicin, Lam et al[9] found that 25% developed HSV infection during their induction period. These also similar to those reported earlier by Lam et al.[9] In the present study, the prevalence of HSV 1 and 2 IgG in the 68 acute leukemia was 47/68 (69%) and in the normal siblings was 16/20 (80%). In addition, in the present study 11.7% cases were positive for HSV-1 and 2 IgM, and 19% cases showed reactivation of the viruses, so those patients may be at higher risk of developing disseminated HSV infection. Acyclovir prophylaxis has been shown to be highly effective for seropositive patients who are undergoing induction chemotherapy for acute leukemia. Dong et al[10] found 10/75 cases of positive herpes virus IgM in blood specimens by using ELISA technique. Acquired immune deficiency accompanying leukemia and lymphoma has been associated with reactivation of EBV. Clinical infectious mononucleosis, presumably secondary to EBV, has been reported to precede or follow ALL, and in most cases is benign. However, there is exception as several reports presented cases of EBV-related lymphoproliferative disorder in children with leukemia, which is a serious and often fatal complication in immunosuppressed patients.[11] Epstein-Barr virus infection is highly prevalent in Egyptian population [27 (66.7%)], while in other study, the prevalence rate was [28 (20%)]. In the present study, the high prevalence of EBV-IgG was found both among leukemic children and normal controls (83% and 95%). This high prevalence may be explained by the low socioeconomic standard, large family size, and overcrowding, which are factors that increase the rate of oral excretion and raise the rate of infection. However, higher percentage of EBV IgM was found among normal control (15%) than leukemic children (4.5%). This may be explained as the inability of the patients to respond immunologically to viral infection. In a study carried out by Manabe et al[12] reported that the prevalence of reactivated EBV infection in children with juvenile myelomonocytic leukemia was

4/18 (22%). Cytomegalovirus infections have always been extremely widespread, with high infection rates occurring in third world countries (up to 100%) than in western world (50-80%).[13] In the present study, such as EBV, there was no difference between leukemic children and their controls in overall IgG seropositivity for CMV (100%). The age-specific prevalence rates of IgG antibodies to EBV and CMV rose rapidly after birth to reach a value of over 90% by fourth year of life. The early median age of virus infection (1.4 years for EBV and <1 year for CMV) indicate that primary infection with these viruses occurs early in life. In contrast, age specific prevalence rates of IgG to VZV and HSV rose gradually after birth to attain maximal values of only 83% HSV in the 15-25 year of age group, the median age of infection was delayed (8.2 years for HSV).[14] This also was observed in our results as not only prevalence of HSV-1 and 2 IgG increased form 54% in children <5 years to 77% in children >5 years, but also reactivation of HSV-1 and 2 increased with age from 3% in children <5 years to 30% in children >10 years. This in contrast to seroprevalence of CMV(100%) and EBV (95%) in children <5 years. Herpes simplex virus types 1 and 2 seropositivity is associated with female gender, non-white ethnicity, and a history of sexually transmitted disease.[15] In the present study, there was no difference in seropositivity to any of the herpes viruses studied as they are children and not sexually active. Leukemic children with granulocytopenia are at higher risk of herpes virus reactivation and serious diseases.[16] In the present study, there was no difference in the seropositivity between children with granulocytopenia (17%) and those without granulocytopenia (82%), prophylactic administration of acyclovir to HSV Ab positive patients during neutropenia might reduce not only morbidity related to mucosal ulceration but also incidence of antibiotic resistant fever.[17] In the present study, 29/68 (42%) of the leukemic patients showed abnormal ALT level and it was observed that most cases with positive IgM for HSV-1 and 2 (6/8 [75%]), and positive EBV IgM (2/3 [66.7%]) had abnormal ALT level, which indicates that these viral infections could be associated with hepatitis. They reported 5 cases of fulminant hepatitis due to HSV among patients admitted to transplantation institute.[18] In another report, Julian et al[19] described an adolescent diagnosed with Hodgkin disease who developed fatal hepatic failure secondary to acute HSV.[19] Peterson et al[20] observed that abnormal liver function tests and leukopenia were common among renal allograft recipients with overt CMV disease, and the degree of abnormality correlated with severity of the CMV disease.

In conclusion, the data indicate a higher exposure to HSV-1 and 2 both primary infection and reactivation among ALL children. Therefore, acyclovir prophylaxis could be highly effective for seropositive patients who are undergoing induction chemotherapy for acute leukemia.

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