Plasma of Acute Lymphoblastic Leukemia Patients React to the Culture of a Mycovirus Containing *Aspergillus flavus*

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Summary: Acute lymphoblastic leukemia (ALL) is the most common cancer in children and is also seen in adults. Currently, no plasma-based test for the detection of ALL is available. We have cultured the home of a patient with ALL and isolated a mycovirus containing *Aspergillus flavus*. This culture was subjected to electron microscopy, purification, and mass spectrometry. Using enzyme-linked immunosorbent assay technique, plasma of patients with ALL and long-term survivors of this disease were tested for antibodies, utilizing supernatant of the culture of this organism. The results were compared with 3 groups of controls, including healthy individuals, patients with sickle cell disease, and solid tumors. Using electron microscopy, the isolated *A. flavus* contained mycovirus particles. In chemical analysis, this organism did not produce any aflatoxin. Using an enzyme-linked immunosorbent assay technique, the supernatant of the culture of the mycovirus containing *A. flavus* could differentiate ALL patients from each group of controls ($P < 0.001$). These studies provide a new technique for the detection of ALL and may add information for future research regarding leukemogenesis.

Key Words: acute lymphoblastic leukemia, *Aspergillus flavus*, mycovirus, ELISA test, leukemogenesis

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Acute lymphoblastic leukemia (ALL), with an incidence of 3 to 4/100,000 pediatric population, constitutes the most common childhood cancer, accounting for ~20% of all malignant disorders in this age group.[1] This disease also occurs in adults, albeit with a much lesser frequency.[2] Currently, the diagnosis of leukemia is based on cellular recognition of the disease, done mainly by obtaining bone marrow aspiration for pathology, flow cytometric examination, and cytogenetic analysis. No plasma-based diagnostic tool to screen for, or diagnose this disease is available.

We have isolated *Aspergillus flavus* from the home of a patient with ALL and have extensively evaluated this isolate which by electron microscopy evaluation contains mycovirus particles. The mycovirus particles were found within the body of the organism and culture supernatant. Chemical analysis of our isolated mycovirus containing *A. flavus* revealed no evidence of aflatoxin production. Fungi, as virus hosts, via a virus/virus and virus/host interactions, can block the production of aflatoxin which may be the case with our isolate. The studies described here reveal that indirect enzyme-linked immunosorbent assay (ELISA) technique, using the supernatant culture of this mycovirus containing *Aspergillus flavus* (SAF) can identify and distinguish plasma of ALL patients in full remission from normal individuals or patients with sickle cell disease (SCD) and those with solid tumors.

Fungi are widely distributed in the environment and their products can be carcinogenic. Prior reports of isolation of fungi in residences of individuals with leukemia are available but their carcinogenic effects were attributed to immunosuppression and aflatoxin exposure, not their direct effect as we have shown in our studies.

Currently, no known plasma-based techniques for identification of individuals with ALL or diffuse lymphomas in remission are available. These findings may have the potential for diagnosis of, or screening for, ALL. Furthermore, by extension, it may provide some information for future research on the pathogenesis of this disease.

MATERIALS AND METHODS

Participants

For the performance of the plasma-based studies, according to an Institutional Review Board approved protocol, informed written consent from parents of patients under 18 and individuals over 18 years of age was obtained. Informed assent was also required for patients ages 12 to 17 years. As a protocol requirement, all participants had to have no history of fungal infection. The ALL patient population consisted of a group of 40 individuals with precursor B-cell ALL. No selection based on risk factors or prognosis was made. For controls, to assure specificity, 3 separate groups were utilized. The first group was that of 20 healthy blood donors without any disease. In this group, discarded blood from the blood bank was used. The second group of controls consisted of 26 patients with SCD undergoing manual

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Under contracts, protein purification and mass spectrometry were performed by Dr Jinyi Zhu, of Protein Production Core facilities and Electron Microscopy by Amanda Garces of Advanced Imaging Core Laboratories at the University of South Florida.
Studies described are a part of patents registered in the United States, European Union, Japan, Russia, and elsewhere. C.K.T. is the patent holder.
C.K.T.: original idea, collection, and isolation of mycovirus containing *Aspergillus flavus*, design of the studies, preliminary laboratory work, supervision of the project and writing of the manuscript. A.B.: performed ELISA tests, coincubation studies with EBV, FPLC separation, collection, and analysis of the data. E.S.: supervised confirmatory studies and assisted with the preparation of the manuscript. A.I.A.: assisted with the preparation of graphs and statistics. S.N.: performed confirmatory studies. J.J.P.: performed confirmatory studies and assisted with graphics. A.N.A.: assisted with confirmatory studies. M.V.J.: assisted with IRB approval for confirmatory studies. S.P.: assisted with the patient recruitment and preparation of samples for confirmatory studies. F.M.: performed the initial technical work.
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partial exchange transfusion. Patients with SCD undergoing partial exchange transfusion were selected as a control group since some of these patients require periodical partial exchange transfusion thus providing an available source of blood. In this group, to avoid any mixture of patient and donor blood, only the first blood drawing was utilized. The third group of controls was 30 patients with a variety of nonlymphatic solid tumors. For the latter 2 groups of controls informed consents, as described above, were obtained. Approximately 15 to 20 mL of blood from each individual was collected in 500 U of preservative-free heparin. To obtain plasma, a portion of the collected blood was centrifuged at 2000 rpm for 12 minutes at 4°C. Collected plasma was used immediately or kept frozen at −80°C for future use. The remaining blood was used for cellular investigations.

A. flavus

As a part of surveillance of the home environment of a patient with ALL, parents were given a petri dish of Sabouraud Dextrose Agar media to open in the bedroom of the patient for 10 minutes and return it for fungal evaluation. The patient had ALL in remission and no prior history of fungal infection or other unique characteristics. Axenic culture was isolated from the plate. For the present study, only 1 culture containing growth of *A. flavus* was used throughout all the described experiments. The isolated *A. flavus* was cultured in a glass bottle with an underlayer of 1% solid agar and an overlayer of 3.5 g% Czapek-Dox broth (Difco; Becton Dickinson, Sparks, MD). The culture of this isolate was incubated at 37°C in air, with removal of the supernatant and replacement with Czapek-Dox broth approximately every 4 weeks. The supernatant of the culture of this mycovirus containing *A. flavus* was filtered through a 0.25 μm filter (Corning Inc., Corning, NY), stored at 4°C up to 4 weeks and concentrated before its utilization using Amicon Ultra-15 centrifugal filter units (EMD Millipore, Burlington, MA). The protein concentration of this filtrate was adjusted to ∼3 mg/mL before its use.

To evaluate the *A. flavus* for mycoviruses, both the fungal growth and supernatant of the culture were analyzed for viral contents by electron microscopy. The grown organism was placed into resin blocks suitable for ultramicrotomy sectioning at 100 nm or less, collected on copper grids and contrast-enhanced with uranyl acetate before transmission electron microscopy observation.

To separate protein content of the supernatant of the culture of the mycovirus containing *A. flavus* mainly according to the size and charge, fast protein liquid chromatography (FPLC) (AKTA system; GE, Aurora, OH) with standard techniques was utilized. Supernatant of the culture of *A. flavus* alone, Epstein-Barr virus (EBV) alone, or their coculture was individually collected and analyzed using FPLC. Each resulting peak was collected and evaluated separately, and the results were compared.

For protein purification, SAF was buffer exchanged into 50 mM Tris (pH 8.0) containing 1 mM EDTA and 1 mM DTT via HiPrep 26/10 Desalting column (GE Healthcare, Uppsala, Sweden), before it was loaded onto a HiTrap Q HP column (GE Healthcare). Target protein was purified by a 20-column volume linear gradient of 0 to 500 mM NaCl. Fractions containing target protein were concentrated and loaded onto a Superdex 200 10/300 GL (GE Healthcare, Chicago, IL) which was equilibrated with 50 mM Tris (pH 7.5) containing 300 mM NaCl and 1 mM EDTA. Fractions were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the corresponding band was confirmed by mass spectrometry.

To evaluate the possible effects of EBV, SAF culture alone or after coincubation of *A. flavus* culture with EBV for 7 days, were used. The EBV source was a commercially available EBV, which was adjusted to 2×10^6 plaque-forming units/mL. As a control for EBV, avian leukosis virus (ALV) was obtained from commercial sources and used at 2×10^6 plaque-forming units/mL. ELISA tests in an identical fashion as described below, was performed in 10 ALL patients and controls using ALV substituting for EBV. This group of ALL patients included 5 male and 5 female patients, 6 of whom were white, 3 Hispanic and 1 black.

To detect antibodies in plasma samples, a qualitative indirect ELISA assay technique was performed using Corning 96 well plates. The plates were coated with filtered and concentrated SAF for a total volume of 100 μL in each well and incubated at 4°C for ∼24 hours. Post concentration, the approximate protein content of SAF was adjusted to ∼3 mg/mL. For negative controls, RPMI media was used. After incubation, the plates were washed thrice in blocking buffer (2% bovine serum albumin in phosphate-buffered saline) for 5 minutes and then incubated in blocking buffer for 2 hours at 37°C. In each well, 100 μL plasma samples of normal or ALL patients were added. The plates were incubated at room temperature for 2 hours. Goat anti-human secondary antibody conjugated with alkaline phosphatase (Promega, Madison, WI) was used in the dilution of 1:5000 in blocking buffer and 100 μL was added in each well and incubated at room temperature for 2 hours. The plates were washed 5 times with phosphate-buffered saline/0.1% Tween 20 (PBST), pH 7.4, for 3 minutes by placing the plate on low speed shaker after each step of incubation. After the washing steps of the secondary antibody, 100 μL of substrate 1 step pNPP (Thermo Fisher Scientific, Lafayette, CO) was added to each well and allowed to develop color for 30 minutes. The reaction was stopped by the addition of 50 μL of 2 N NaOH in all the wells. Absorbance was measured at 405 nm using Biotek Synergy 2 microplate reader. All tests were performed in triplicate.

Statistical Analysis

Statistical analysis was done using the Student *t* test. All ELISA results were expressed as state OD at 405 nm± SD. A *P*-value ≤ 0.05 was considered as statistically significant.

RESULTS

All patients and controls had consented/assented to participate in the study. None of the patients participating in this study had a history of fungal infection.

The ALL patients' ages ranged from 5 to 23 years. This group consisted of 26 white, 9 Hispanic, 4 black, and 1 individual of Asian ancestry. ALL patients were in full remission, and all but 5 long-term survivors, were actively receiving chemotherapy. Plasma of 3 newly diagnosed patients had a reaction to the SAF similar to those in remission, but for uniformity of the data, only remission patients are included in this report. Off therapy patients had not received chemotherapy for periods ranging from 1 to 3 years.

The first group of controls consisted of 20 healthy blood donors. The second group was that of 26 Black patients with SCD with ages ranging from 9 to 16 years undergoing partial exchange transfusion. The third control group were 17 white, 9 Hispanic, and 4 black patients with a variety of solid tumors.

Electron microscopy of the *A. flavus* revealed the existence of virus-like particles within this organism. The sizes of the particles observed ranged between 30 and 50 nm and were found in

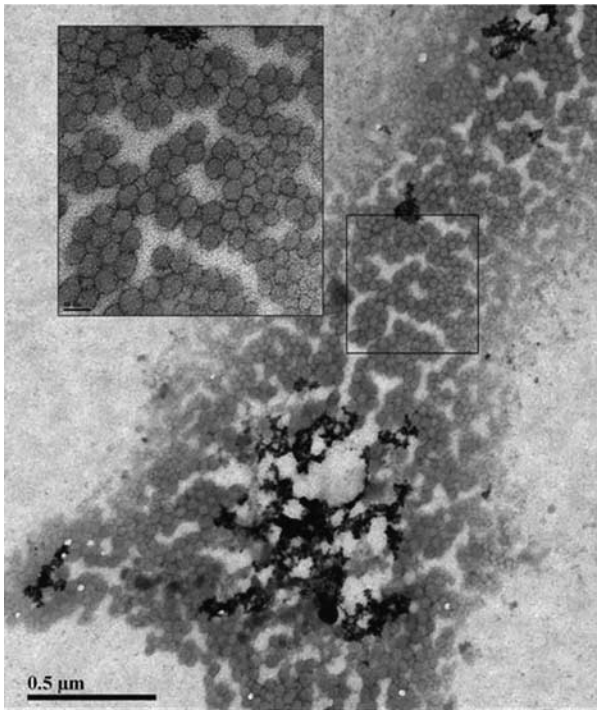

FIGURE 1. Virus-like particles collected from supernatant of glutaraldehyde-fixed suspension culture of *Aspergillus flavus*. The average diameter of particles in this micrograph is 34 nm which represents the most prevalent size seen. Inset (50 nm scale) shows enlarged detail of area in square. Transmission electron microscopy micrograph of formvar-coated copper grid preparation. Contrast-enhanced with aqueous uranyl acetate.

single or aggregate form, with or without patent dense cores. These particles were seen in both, the body of the organism and the culture supernatant (Fig. 1). Smaller and larger particles ranging from 20 and 25 nm and 60 and 80 nm containing dense cores were seen in the hyphae.

The FPLC separation of the supernatant of the culture of mycovirus containing *A. flavus* produced 3 separate peaks (Fig. 2). The FPLC evaluation of EBV revealed 2 peaks (Fig. 2). Combination of the culture of *A. flavus* and EBV produced a pattern which was different than that of FPLC analysis of each culture alone (Fig. 2).

The major probable constituents of the products of mycovirus containing *A. flavus* used in the experiments, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and confirmed by mass spectrometry, were: dipeptidyl peptidase 5, catalase B, serotransferrin, glyceraldehyde-3-phosphate dehydrogenase, acetylxylan esterase A, beta-glucosidase A, leucine aminopeptidase 2, beta-galactosidase A, dipeptidyl peptidase 4, glucan endo 1,3-beta-glucosidase C, glucoamylase, mannosyl-oligosaccharide alpha-1,2-mannosidase 1B, beta-glucosidase M, glucan endo 1,6-beta-glucosidase B, beta-glucosidase G, alpha-galactosidase A, and alpha-galactosidase C.

With the ELISA technique, using filtered unseparated SAF, there was a significant difference between plasma of patients with ALL, that is, 2.07 ± 0.26 SD versus controls including normal individuals (1.31 ± 0.11 SD, $P < 0.001$), or patients with SCD (1.43 ± 0.22 SD, $P < 0.001$), or solid tumors (1.33 ± 1.32 SD, $P < 0.001$) (Fig. 3). The finding was irrespective of time since the diagnosis of ALL and duration of the disease. These differences were not observed comparing the results from plasma of normal individuals to patients with SCD and those with solid tumors ($P > 0.05$). Thus, while SAF could differentiate plasma of patients with ALL from controls, it could not distinguish plasma of

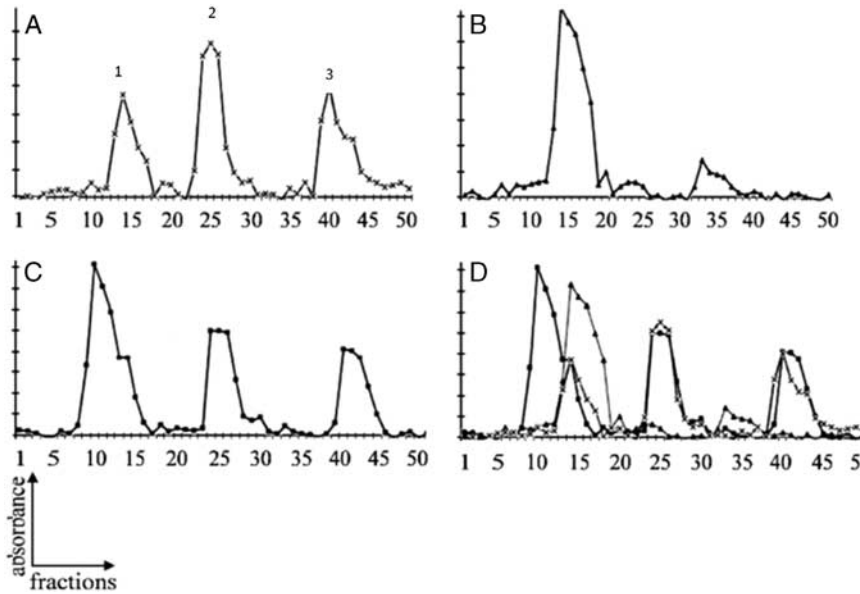

FIGURE 2. Fast protein liquid chromatography fractionation of (A) supernatant of the culture of a mycovirus containing *Aspergillus flavus*, isolated from the home of a patient with acute lymphoblastic leukemia, (B) Epstein-Barr virus (EBV). C, Supernatant of the culture of the mycovirus containing *A. flavus* cocultured with EBV. D, Superimposed peaks of A, B, and C. Peaks obtained with coculture of the supernatant of the culture of *A. flavus* with EBV are different than each organism cultured alone.

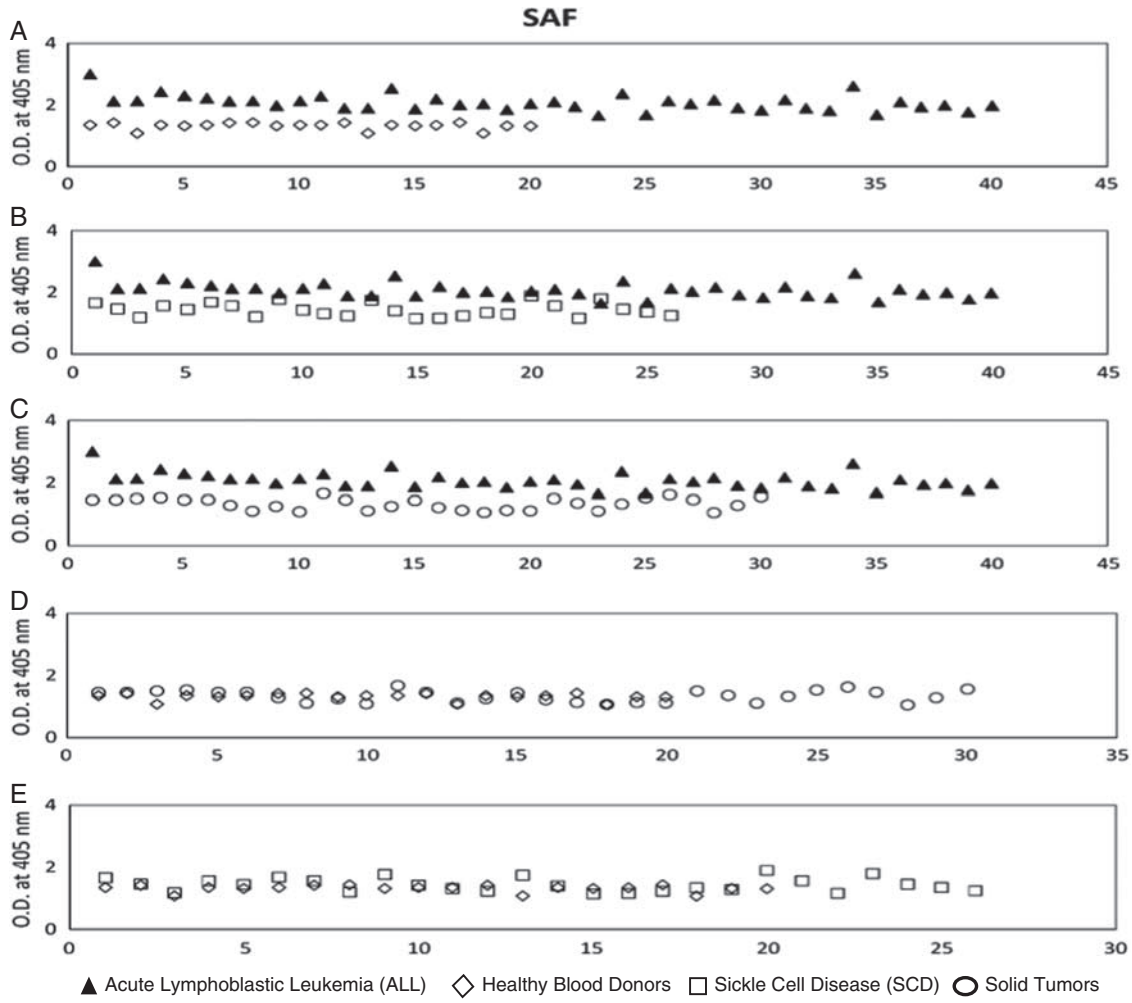

FIGURE 3. Reactivity of the supernatant of mycovirus containing *Aspergillus flavus* (SAF) using indirect enzyme-linked immunosorbent assay technique and plasma of patients with ALL as compared with normal blood donors ($P < 0.001$) (A), patients with SCD ($P < 0.001$) (B), and solid tumors ($P < 0.001$) (C). While SAF could differentiate plasma of patients with ALL from controls, it did not differentiate plasma of healthy blood donors from those with solid tumors (D) or with SCD (E) ($P > 05$).

healthy blood donors from those of patients with SCD or with solid tumors (Fig. 3). There were only a small number of blacks and Hispanics in the cohort of the patients tested, thus excluding meaningful statistical comparisons based on these parameters. However, no gross differences based on race and ethnic background were noted. EBV could differentiate ALL patients (2.04 ± 0.21 SD) from normal individuals (1.33 ± 0.07 SD, $P < 0.001$) and those with SCD (1.4 ± 0.13, $P < 0.001$) or solid tumors (1.25 ± 0.10, $P < 0.001$) (Fig. 4). However, addition of EBV to SAF did not enhance the activity of the unseparated supernatant of the culture of *A. flavus* (Fig. 5). The results for the combination of SAF+EBV for plasma of patients with ALL was 2.04 ± 0.15 SD as compared with 1.25 ± 0.15 SD for normal individuals ($P < 0.001$), 1.39 ± 0.15 SD for patients with SCD ($P < 0.001$), and 1.32 ± 0.17 SD for those with solid tumors ($P < 0.001$).These results are not statistically different than those seen by SAF or EBV alone. ALV, which was used as a control for EBV, failed to distinguish ALL patients from the control group including normal individuals, patients with SCD and nonlymphatic solid tumors ($P > 0.05$) (Fig. 6).

When peaks separated by FPLC were tested with indirect ELISA technique, using plasma of ALL patients as compared with controls, peak 1 proved to have the highest activity among the 3 separated peaks ($P \leq 0.001$) (Fig. 7). Indeed, the activity of peak 1 was superior to that of unseparated whole SAF. Peak 3 had a marginal effect. Peak 2 had no significant effect ($P > 0.05$). None of the peaks separated by FPLC reacted to the plasma of the normal individuals or those of SCD or solid tumor patients ($P > 0.05$) (Fig. 7).

DISCUSSION

ALL with an overall incidence of 1.7/100,000 population, occurs both, in adults and children.[1,2] However, the incidence is much higher in the pediatric age group, amounting to 3.4/100,000 population.[1] The finding of an antibody present in patients with ALL, and not controls, may be of significance and may provide a plasma-based test for screening and diagnosis of ALL. Furthermore, it provides a consistent organism involved in this disease. Of note

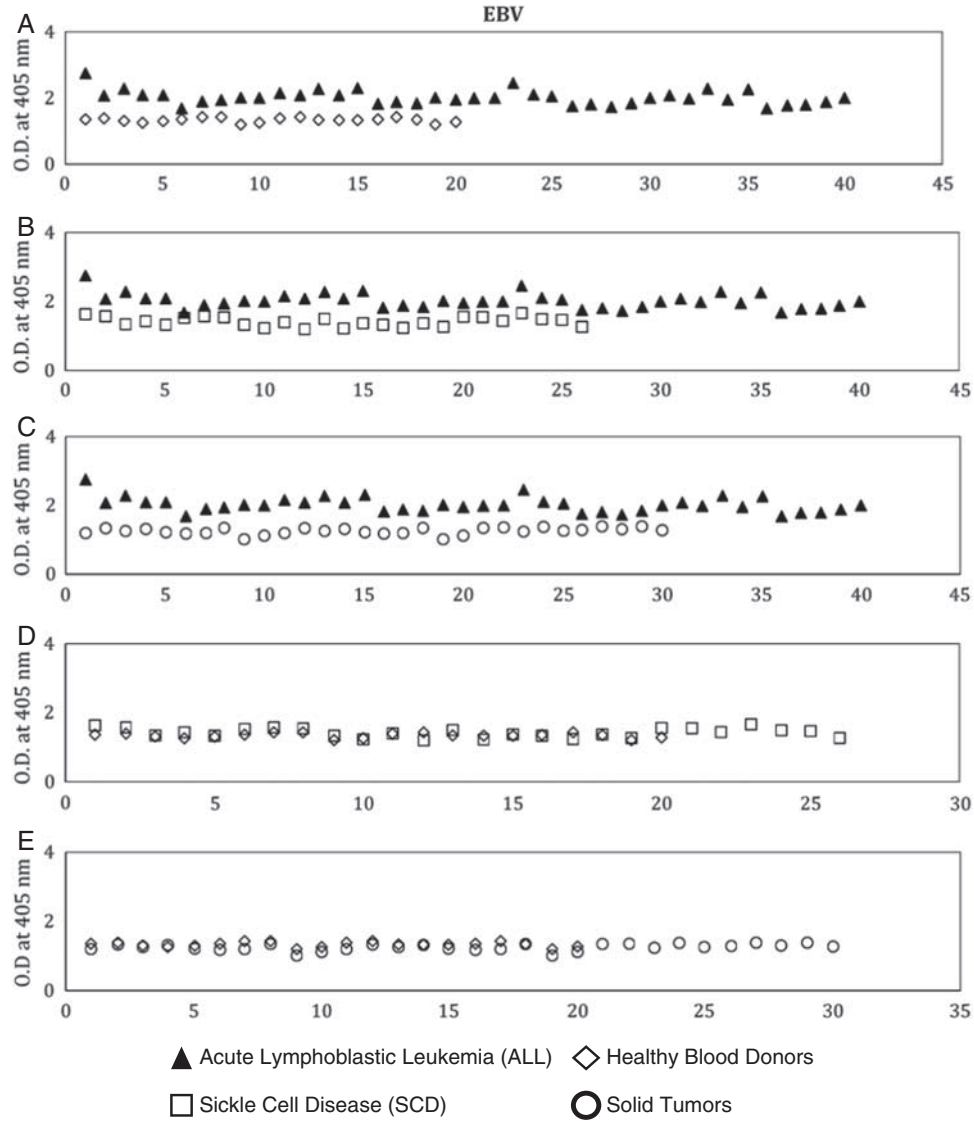

FIGURE 4. Reactivity of the supernatant of the mycovirus containing *Aspergillus Flavus* (SAF) incubated with Epstein-Barr virus (EBV) using indirect enzyme-linked immunosorbent assay technique and plasma of patients with ALL as compared with controls ie. A, Healthy blood donors ($P < 0.001$), (B) patients with SCD ($P < 0.001$) (B), and (C) solid tumors ($P < 0.001$). The combination did not significantly alter the results obtained with SAF alone. While the combination could differentiate plasma of patients with ALL from controls, it did not differentiate plasma of healthy blood donors from those with solid tumors (D) or those with SCD (E) ($P > 0.05$).

is the presence of this antibody in the plasma of the patients regardless of the time of diagnosis and duration of the disease. The findings also may provide support for the recently proposed modified 2-hit theory.[3] This theory concerns genetic factors as well as prenatal initiation lesion and abnormal immune response to 1 or more common infections as triggers for the development of ALL.[3] While various genomic profiling, including genome-wide association studies, have revealed evidence of multiple gene variant involvement as risk factors for ALL,[4–7] no specific group of infections so far has been identified.

Despite significant progress made in the treatment of ALL, the etiology of this disease is subject to speculation. Numerous factors contributing to the development of ALL have been suggested. These include genetic and epigenetic factors and defined medical disorders,[8–16] exposure to ionizing radiation,[17–20] magnetic fields,[21,22] various parental, family and social factors,[23–27] birth history,[28–30] nutritional variables,[31–33] early exposure to infections and day care attendance,[34–36] allergies or aberrant immune responses,[37,38] exposure to chemicals,[39] preexisting conditions, genetic variations and disorders such as myelodysplastic syndrome.[40–43] Socioeconomic factors and population mixing causing an increased in occurrence of ALL has been reported, but not uniformly accepted.[44,45]

Generally, certain infectious agents, including viruses, bacteria, and parasites are reported to increase risk of cancer.[46–50] Recent data reveals substantial microbial contribution to some types of cancer. Examination of the whole-genome and the whole transcriptome sequencing studies in Cancer Genome Atlas (TCGA) in untreated oncology patients has revealed unique microbial signatures

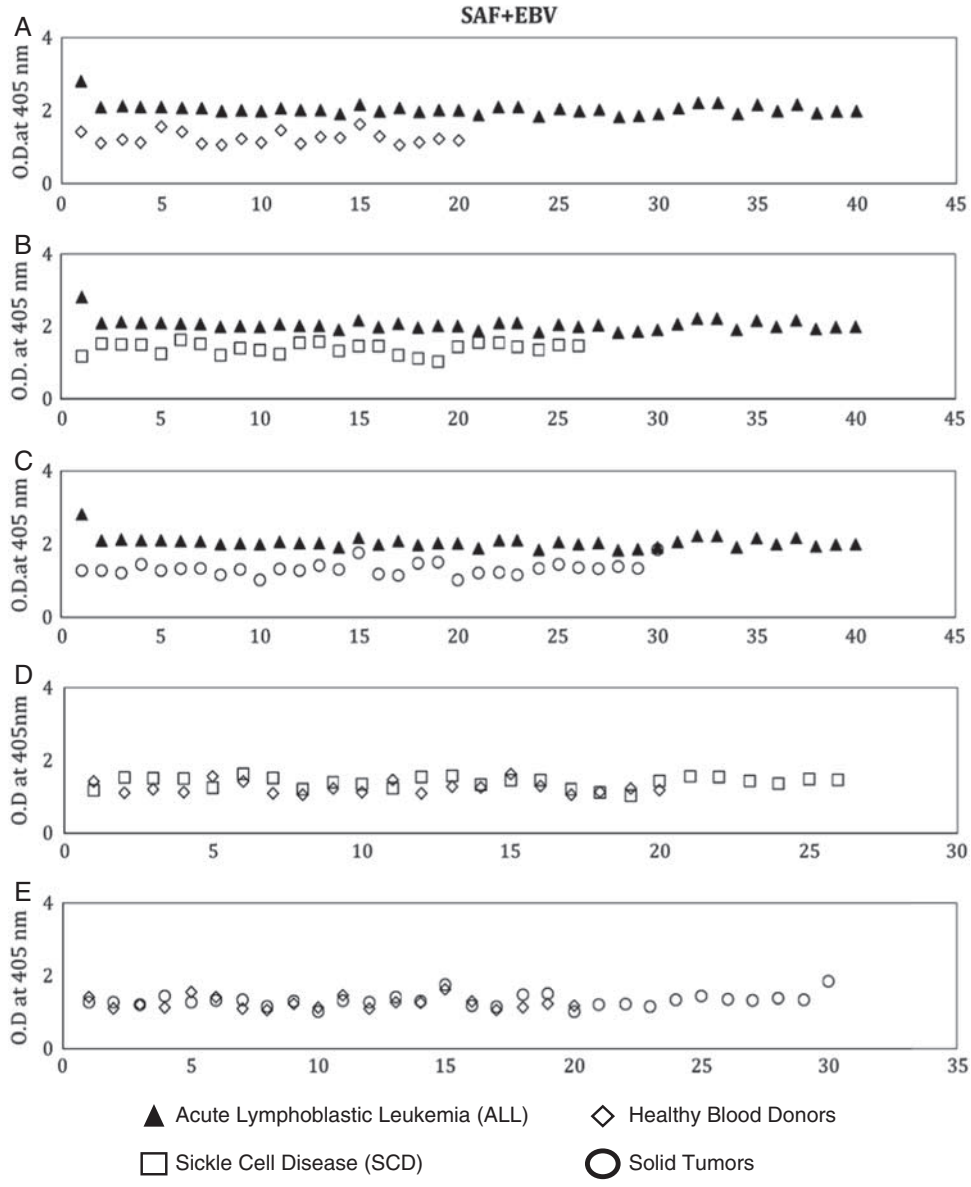

FIGURE 5. Reactivity of the supernatant of a mycovirus containing *Aspergillus flavus* (SAF) incubated with Epstein-Barr virus (EBV) to the plasma of patients with ALL using indirect enzyme-linked immunosorbent assay technique as compared with normal blood donors ($P < 0.001$) (A), patients with SCD ($P < 0.001$) (B), and solid tumors ($P < 0.001$) (C). While a coincubated SAF and EBV could differentiate plasma of patients with ALL from controls, it did not differentiate plasma of healthy blood donors from those with solid tumors (D) or with SCD (E) ($P > 0.05$).

in tissue and blood within and between most major types of cancer.[50] Infections have been suspected to have a role in the development of ALL, an assumption without the availability of a consistent agent.[3,35,36,51–54] The association of EBV and Burkitt's lymphoma in the endemic area of Eastern Africa is well documented. However, lack of universal application of the finding, characteristic 8;14 chromosomal translocation resulting in constitutive activation of c-Myc oncogene, variation in viral gene expression in subgroups of patients, effects of EBV oncoproteins, p53 mutations and a host of other factors have complicated this association.[53] The effect of early exposure to EBV during the first 2 years of life, resulting in a serologic response, has been explored.[53] In our experiments, while EBV alone could distinguish ALL patients from controls, its addition to SAF failed to change the effects of SAF in distinguishing ALL patients from controls. Of interest is that in FPLC analysis, the combination of the culture of *A. flavus* and EBV produced a pattern which was different from FPLC analysis of each culture alone. Substitution of ALV for EBV, which was used as a control, failed to distinguish ALL patients from controls or alter the results.

In FPLC-separated SAF, peak 1 had the most activity in ELISA test, differentiating ALL from controls. This significantly exceeded that of unseparated SAF. Therefore, this peak may have potential to be utilized as a reagent in ELISA test for screening or diagnosis of ALL.

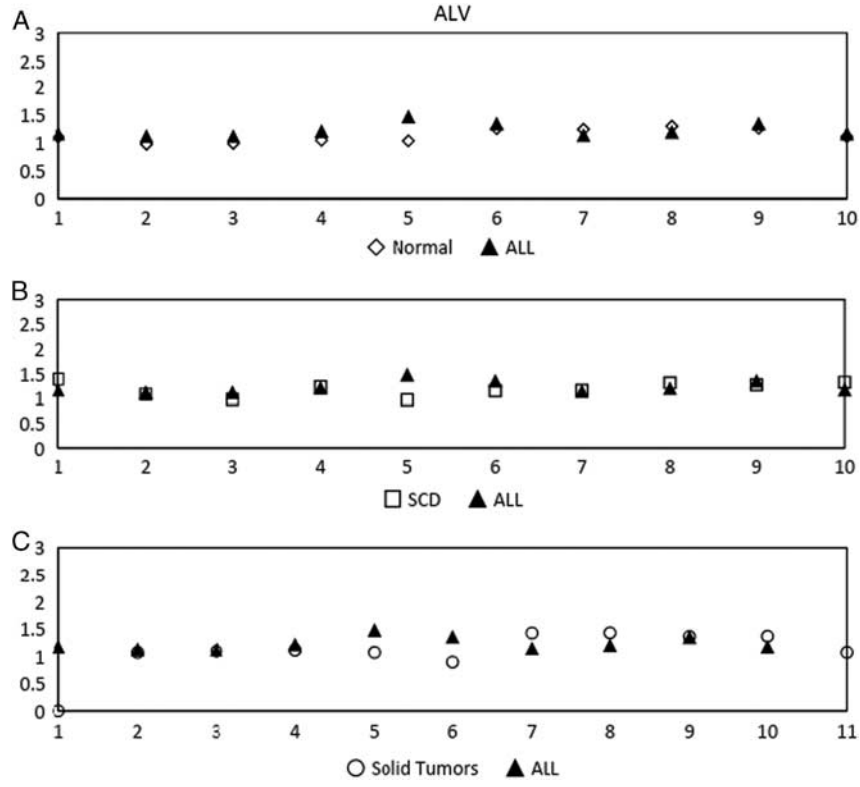

FIGURE 6. Reactivity of avian leukosis virus (ALV) in patients with acute lymphoblastic leukemia (ALL) as compared with (A) normal individuals ($P > 0.05$), (B) patients with sickle cell disease (SCD) ($P > 0.05$), and (C) patients solid tumors ($P > 0.05$) using enzyme-linked immunosorbent assay technique.

Our findings introduce supernatant of a mycovirus containing *A. flavus* as a test for possible screening and diagnostic test for ALL. The fact that plasma of patients with ALL reacted to this organism, as shown by ELISA technique, is of significance. This along with our findings that exposure of the peripheral blood mononuclear cells of ALL patients in remission to SAF results in the genetic and ALL-like cell surface marker changes (C.K.T., A.B., E.S., J.J.P., A.N.A., S.P., F.M., Unpublished

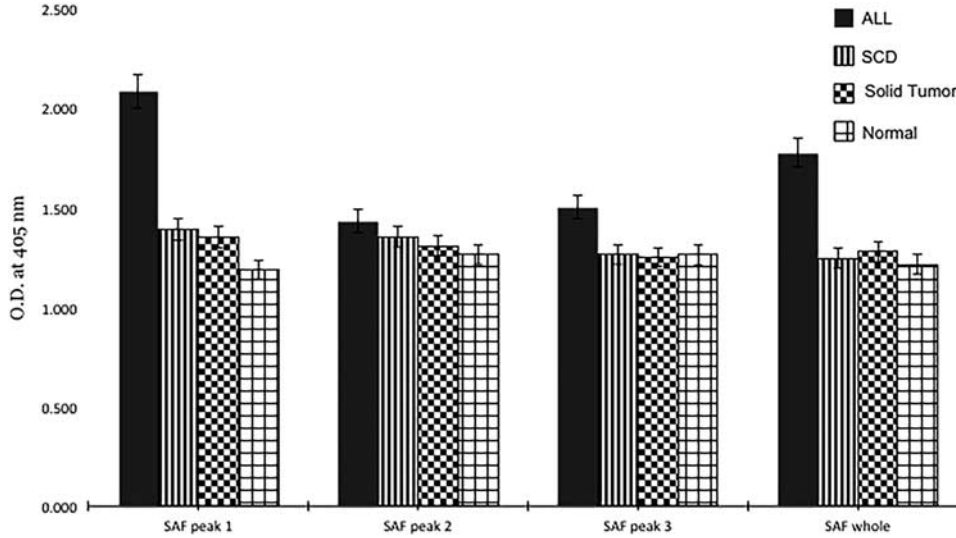

FIGURE 7. Reactivity of the whole supernantant of the culture of mycovirus containing *Aspergillus flavus* (SAF) and 3 peaks obtained by fast protein liquid chromatography, using an indirect enzyme-linked immunosorbent assay technique. Each individual peak is tested with the plasma of patients with acute lymphoblastic leukemia (ALL), sickle cell disease (SCD), solid tumors, and normal patients. SAF peak 1 showed the most reactivity with the ALL plasma using indirect enzyme-linked immunosorbent assay technique as compared with the SCD, individuals with solid tumors, and normal group ($P < 0.001$).

data) may provide mycovirus containing *A. flavus* as a consistent infection agent, with and without EBV, to be explored for future research in the process of leukemogenesis.

Fungal organisms in general, and *Aspergillus* species in particular, are widely distributed in the environment. Carcinogenic effects of fungi are well recognized[55] but the full mechanism of their action is not entirely clear. Mycotoxins, including aflatoxins, ochratoxin A, fumonisins, certain trichothecenes, and zearalenone, which are produced by fungal agents, are known to be carcinogens.[55] In the electron microscopy evaluation, the *A. flavus* used in our experiments proved to contain mycovirus particles. Double-stranded RNA infections are known to occur and remain in *Aspergillus* section *Flavi*, regardless of the length of being in culture as is the case in our isolate.[56] Chemical analysis of the culture of the mycovirus containing *A. flavus* used in our described experiments, revealed no evidence of aflatoxin production. Some studies have shown that fungi, as virus hosts, provide a unique platform for virus/virus and virus/host interactions[57] and can block the production of aflatoxin.[58,59] This appears to be the case in the *A. flavus* utilized in our studies. Few reports of fungal isolation from residences of leukemic patients are published,[60–63] but their carcinogenic impacts were attributed to immunosuppression,[62,63] and aflatoxin exposure,[61] not direct effect as we have shown in our studies. In one report, sera from 36 patients with cancer, 15 of whom had leukemia or lymphoid malignancy, utilizing a modified microimmunodiffiusion technique, the supernatant of the culture of *Aspergillus* produced 30% precipitation in cancer patients versus 6% in controls.[62] This was attributed to aflatoxin. As noted before, in multiple tests our mycovirus infected *A. flavus* did not produce any aflatoxin.

It is of interest that in the present study, mycovirus containing *A. flavus* was isolated from the home of a patient with ALL. With ELISA technique, it was possible to distinguish patients with ALL in remission from "normal" controls and those with solid tumors. These studies present a preliminary information for future research and for a plasma-based test for screening and diagnosis of ALL. A significant research and further evaluation to better understand the mechanism of action of SAF is needed.

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