The putative role of transforming viruses in childhood acute lymphoblastic leukemia

Epidemiological evidence suggests that infection is involved in the etiology of common acute lymphoblastic leukemia, either by stimulating an inappropriate immune response or in the form of a classical transforming agent. In an attempt to elucidate the role that infection is playing in this disease, we used representational difference analysis (RDA) to examine tumor samples for the presence of exogenous genomes. Twenty RDA experiments were carried out, using four different restriction enzymes, but no exogenous sequences were identified within leukemic cells. These results suggest that it is unlikely that a single, direct transforming agent is involved in the pathogenesis of common acute lymphoblastic leukemia.

Key words: etiology, genomes, subtraction, pathogen, leukemia.

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hildhood acute lymphoblastic leukemia (ALL) comprises several entities with different cancer cell phenotypes and age-specific incidence curves, which are likely to be etiologically distinct. Common acute lymphoblastic leukemia (cALL), in which the leukemic cell is a B-cell precursor, accounts for the peak in incidence rate seen between the ages of 2 and 5 years in areas of high socio-economic status. For cALL, there is now unambiguous molecular evidence that, in most cases, a pre-leukemic state is initiated in utero. The MLL-AF4 and TEL-AML1 fusion genes or a hyperdiploid karyotype can be detected at birth in the blood of children who subsequently go on to develop ALL. These identical TEL-AML1 fusions have also been detected in monozygotic twins with concordant leukemia; however, concordance of cALL in monozygotic twins is modest (~10%) suggesting that post-natal events, i.e., exposures and secondary events, are required for the development of overt leukemia.

The idea that infectious agents may play some role in the development of childhood leukemia is long-standing and plausible. Two distinct, but not mutually exclusive, models have been proposed based on time trends or geographic variation in incidence or clustering of cases. The broad association between cALL and socio-economic development has led to the suggestion that cALL may occur as a consequence of delayed exposure in infancy to an infectious agent or agents. An abnormal or dysregulated immunological response to post-natal infection, promoting secondary genetic events and leukemia development, is the key feature of this model. The putative agent(s) may be viral or bacterial, but the mechanism of action is indirect or non-transforming.

The second model predicts that childhood leukemia occurs as a rare response to a common transforming virus under certain demographic conditions. It has been shown that influxes of new populations into previously isolated communities, such as occurred during the development of British New Towns during the 1950s, are associated with transiently increased incidences of childhood leukemia. Kinlen postulated that this was due to unrecognized epidemics of infection by an unidentified virus within such communities, and that these epidemics led to the transiently increased incidence of leukemia. Similar studies outside the UK support this hypothesis, as does space-time clustering. We and others have previously investigated the idea that herpesviruses or the polyomaviruses JC and BK might be involved but found no evidence for the presence of viral genomes in ALL cells. Other investigators have retrospectively analyzed patients’ neonatal blood spots (Guthrie cards) for these same viral sequences or parvovirus B19 and reported negative results. To investigate more comprehensively whether DNA genomes of infectious agents are present in leukemic cells, we analyzed a series of cALL cases using the genomic subtractive hybridization technique, representational difference analysis (RDA).

**Design and Methods**

Considerations on the use of RDA in the investigation of CALL. RDA is designed to identify differences between two complex genomes; in the context of this study the difference is predicted to be a DNA genome present in the leukemic cells but not in normal cells from the same individual. The leukemic sample, containing the putative genome or target, is termed the tester and the partner sample is
temed the driver. In these experiments we used leukemic cells from the patient as the tester and, when possible, granulocytes from a remission blood sample as the driver. Pooled DNA extracted from whole blood samples from both parents of the patient was used in cases in which a satisfactory remission sample was not available.

The probability of detecting a genomic fragment of a given pathogen is dependent on the size of the pathogen’s genome and the restriction enzyme used to generate representations. We calculated that a genome of 150 kb or more, such as a herpesvirus genome, would be detected with a probability of 99% following analysis of a single representation defined by three of the four enzymes used in this study. To give a >95% probability of detecting a viral genome of 30 kb, one or two RDAs would have to be performed and for a 9 kb genome three or four RDA would be required, depending on the restriction enzyme used. We, therefore, carried out RDA using representations derived with four different enzymes. According to our original protocol, we intended to perform four RDA on each of five cases (20 RDA); however, there was insufficient DNA from each case to prepare four representations and we therefore required samples from 11 cases to complete these 20 RDA (Table 1).

Case and sample selection. Eleven patients with cALL, aged between 2 and 15 years, were included in this analysis (Table 1). In all cases the leukemic cells fulfilled the phenotypic criteria for the diagnosis of cALL (CD10, CD19, CD79a, FAB L1 or L2) and >90% of the mononuclear cells were blast cells. Following informed consent, pretreatment blood or bone marrow samples and, when possible, remission blood samples were collected. DNA was extracted from mononuclear and granulocyte fractions using standard procedures. Cases and sample details and the RDA performed are shown in Table 1.

Representational difference analysis protocol. In early experiments the original RDA protocol was followed. After evaluation of the results of these experiments, a number of modifications were introduced: the control driver: driver hybridization was replaced with a tester: driver hybridization; small scale pilot experiments were used to determine the optimal number of polymerase chain reaction (PCR) cycles required at each stage to ensure the maintenance of a wide size range of amplified fragments; more efficient and reproducible post-hybridization amplification was achieved by inactivating the mung bean nuclease by phenol chloroform extraction and ethanol precipitation, rather than heat inactivation; and four separate adaptor-primer sets were used rather than the three (R, J and N adaptor-primers) originally described, to reduce the number of illegitimate products generated by annealing of primers to genomic DNA (supplementary Table, online version). The original protocol using three adaptor-primers was used for all analyses with BglII, both original and optimized protocols were used for BamHI analyses and the optimized adaptor-primer protocol was used in all RDA performed using EcoRI and XbaI.

In those experiments in which discrete fragments were identified following three rounds of RDA, the entire third round products, or gel-purified fragments in later experiments, were cloned into pCR®2.1 or pCR®4-TOPO® using the appropriate TA cloning kit (Invitrogen, Paisley, UK), according to the manufacturer’s instructions. Nucleotide sequencing of cloned fragments was carried out using primers derived from standard vector sequences and Big Dye™ terminator sequencing kits (Applied Biosystems, Warrington, UK). Thermal cycling was performed using a GeneAmp PCR System 2400 or 9600 (Applied Biosystems) and, following removal of unincorporated fluorescent nucleotides, products were analyzed on an ABI PRISM™ 310 or 3100 Genetic Analyzer (Applied Biosystems). The sequences were analyzed using the NIX sequence analysis package (UK Human Genome Mapping Project Resource Centre, Cambridge, UK), which simultaneously performs Blast searches against vector, protein, mRNA, expressed sequence tag and high throughput genome sequence (HTG) databases. When the origin of third round fragments could not be determined by sequence and Nix analysis, fragments were labeled with α-32P dCTP by random priming using Redivue™ kits (Amersham Biosciences UK Ltd., Little Chalfont, UK) and hybridized to driver and tester representations of the case under investigation, using standard procedures.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Enzyme</th>
<th>Driver sample*</th>
<th>Control experiment</th>
<th>Difference products*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>F</td>
<td>BamHI</td>
<td>remission</td>
<td>driver: driver</td>
<td>yes</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>M</td>
<td>BamHI</td>
<td>remission</td>
<td>driver: driver</td>
<td>yes</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>M</td>
<td>BamHI</td>
<td>remission</td>
<td>driver: driver</td>
<td>yes</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>F</td>
<td>BamHI</td>
<td>remission</td>
<td>tester: driver</td>
<td>no</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>M</td>
<td>BglII</td>
<td>remission</td>
<td>tester: driver</td>
<td>yes</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>M</td>
<td>BamHI</td>
<td>remission</td>
<td>tester: driver</td>
<td>yes</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>F</td>
<td>EcoRI</td>
<td>parental</td>
<td>tester: driver</td>
<td>smear*</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>F</td>
<td>BamHI</td>
<td>parental</td>
<td>tester: driver</td>
<td>yes</td>
</tr>
<tr>
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<td>M</td>
<td>EcoRI</td>
<td>parental</td>
<td>tester: driver</td>
<td>yes</td>
</tr>
<tr>
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<td>parental</td>
<td>tester: driver</td>
<td>no</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>F</td>
<td>EcoRI</td>
<td>parental</td>
<td>tester: driver</td>
<td>yes</td>
</tr>
</tbody>
</table>

* Driver samples were either DNA extracted from granulocytes from a remission blood sample from the patient or pooled DNA extracted from the whole blood of the parents. Difference products detectable in driver: tester experiment following three rounds of representational difference analysis. In two analyses using EcoRI representations, a smear was observed on ethidium bromide-stained agarose gels following electrophoresis of products obtained following 35 cycles of PCR, all other post-hybridization products were detectable after 25 cycles of PCR and we therefore presume that there were no genuine difference products in these two analyses.

Table 1. Patients’ details and representations analyzed.
Results and Discussion

In order to investigate the hypothesis that a single transforming agent is involved in the pathogenesis of cALL, we investigated samples from children with cALL using RDA. This technique facilitates the identification of differences between two complex DNA genomes and can, therefore, be used to identify exogenous DNA sequences, such as viral genomes. Following validation of the experimental protocol, RDA experiments, utilizing representations generated following digestion with four different restriction enzymes, were performed using cALL samples as testers (Table 1). In 12 of these experiments no difference products were detected after three rounds of RDA. In the remaining eight experiments, distinct fragments were detected in the third round difference products and these were subjected to cloning and sequencing. Sequences of, or related to, viral genomes were not detected and there was no evidence of non-human DNA in these difference products. Further details are presented in Table 1 and Figure 1.

RDA has a proven track record for viral discovery and has the distinct advantage over other molecular methods of virus discovery that no a priori knowledge of the potential infectious agent is required. The method does, however, have certain limitations. First, exogenous sequences must be present at close to single copy level in the tester sample to ensure detection. In the present study the tester samples contained >90% leukemic cells and pilot experiments confirmed that we were able to detect single copy differences between tester and driver samples. Thus, it is unlikely that we have missed an exogenous sequence present in all the tumor cells but we cannot exclude the presence of a pathogen in a small proportion of the tumor cells, or in another cell population, which is contributing to leukemogenesis by an indirect mechanism. Secondly, infectious agents will only be detected if the starting tester representation includes a fragment containing part of the genome of the pathogen. In order to minimize the risk of missing small genomes, we performed RDA with four different enzymes. We predicted that this would give us a >95% chance of detecting a viral genome of >9 kb, such as a retroviral genome. Lastly, RDA relies on differences between two samples, in this case leukemic cells and either remission granulocytes or pooled parental samples. Genomes present in both samples at similar levels would escape detection by RDA. TT viruses have small genomes (~3.7 kb), infect granulocytes and lymphocytes, and show extensive genomic variation and, therefore, could have been missed in this study; however, there is currently no evidence that these viruses are involved in the pathogenesis of childhood ALL.

Epidemiological and genetic data support a role for common infections in childhood ALL. Our failure to identify exogenous viral sequences in ALL cells via a sensitive, generic screen makes it unlikely that direct transformation is involved in most cases. This leaves an indirect, immunological mechanism as a more plausible explanation.
RJ, MG and JMK conceived the study. The study was performed in RJ’s laboratory under the direction of JMK, who also performed all the pilot experiments and most of the analysis. RC, JP and KW performed the benchwork and KSW helped with some of the later analyses. MG and TE provided the samples. JMK, MG and RJ wrote the manuscript with contributions from other authors. The authors declare that they have no potential conflicts of interest.

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