

Short Communication

Adenovirus DNA in Guthrie cards from children who develop acute lymphoblastic leukaemia (ALL)

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BACKGROUND: In search of a proposed viral aetiology of childhood acute lymphoblastic leukaemia (ALL), the common species C adenoviruses were analysed in Guthrie cards.

METHODS: Guthrie cards from 243 children who later developed ALL and from 486 matched controls were collected and analysed by nested polymerase chain reaction for the presence of adenovirus DNA.

RESULTS: Adenovirus DNA was reliably detected from only two subjects, both of whom developed ALL.

CONCLUSION: Adenovirus DNA is detected in Guthrie card samples at too low a frequency to reveal an association between adenovirus and the development of leukaemia.

British Journal of Cancer (2010) **102**, 796–798. doi:10.1038/sj.bjc.6605581 www.bjcancer.com

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Keywords: aetiology; childhood leukaemia; adenovirus; prenatal infection

Acute lymphoblastic leukaemia (ALL) is the most common malignancy in childhood, with an annual incidence of three to four cases per 100 000 children in developed countries. Although the aetiology in over 95% of ALL is unknown (Doll, 1989; Margolin, 1997), it is widely believed that an infectious agent may have an effect in the development of this haematological malignancy. We recently reported that adenovirus DNA was detected in 13 of 51 Guthrie cards from ALL patients, but in only 6 of 47 healthy controls ($P=0.0122$, Fisher's exact test), indicating that adenovirus may be such a causative agent (Gustafsson *et al*, 2007). However, a subsequent study of twice as many paediatric ALL patients in California failed to find adenovirus DNA in the corresponding neonatal blood spots (Vasconcelos *et al*, 2008). This study sought to further examine the possibility of an association between prenatal adenovirus infection and ALL using a larger number of archived Swedish samples from children who later developed ALL and controls without the disease.

MATERIALS AND METHODS

The patient population included 243 Swedish children who were diagnosed with ALL between 1992 and 2006. The mean age was

5.4 years and the median was 4.3 years (range 67 days to 15 years). Patients were randomly selected from the Nordic Society of Paediatric Haematology and Oncology register. Ten of these 243 children were included in our previous study (Gustafsson *et al*, 2007) and two of them were positive for adenovirus DNA in that study. More than half (58%) of the subjects were male and 217 (89%) of the leukaemias were of B-cell lineage. As controls, Guthrie cards from 486 children with no known history of leukaemia were picked two Guthrie cards apart from the patient Guthrie card in the Guthrie card archive, thereby matching for birthplace and birthdate.

The blood from infants that was collected on Guthrie cards at 3–5 days of age was processed as described previously (Barbi *et al*, 1996; Priftakis *et al*, 2003), except that a total of four punches were harvested from each card compared with three punches in the previous study. This study was approved by the Ethics Committee at Karolinska Institutet and by the Institutional Review Board of Emory University.

The material recovered from four 3-mm punches from a Guthrie card represented approximately 12 μ l of blood containing about 180 000 leukocytes and 120 000 lymphocytes in a final volume of 60–80 μ l. Samples were first tested for the presence of DNA by real-time polymerase chain reaction (PCR) for the human albumin gene (Laurendeau *et al*, 1999; Desire *et al*, 2001), using 5 μ l of material from each sample. The sensitivity of the method was 10 copies per reaction. Positive samples were coded and sent to Atlanta, where they were screened for the presence of species C adenovirus DNA by nested PCR (nPCR-2 in Garnett *et al*, 2009) using primers P11–P14, which were designed to amplify a

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Revised 6 January 2010; accepted 25 January 2010

conserved region of the species C adenovirus hexon gene corresponding to nucleotides 18 838–19 205 of Ad2 (GenBank accession no. BK000407). Samples having one or more positive reactions with nPCR-2 were then re-tested in duplicate with nPCR-2 and with another nested PCR assay (nPCR-1 in Garnett *et al*, 2009) using primers P7–P10 derived from the Ad2 hexon sequence, to amplify nucleotides 20 721–21 572. Both adenovirus-specific PCR assays routinely detect fewer than five copies of the viral genome. Products of nPCR-1 from samples testing positive by both reactions were sequenced at Eurofins MWG Operon (Huntsville, AL, USA) to confirm the adenovirus serotype. The sequences were aligned by Lasergene software (DNASStar, Inc., Madison, WI, USA) and compared with the sequences from species C adenoviruses in GenBank.

RESULTS

DNA was successfully extracted from the Guthrie cards of 243 patients and from 484 of 486 controls. Two of the control samples were negative for the albumin gene by real-time PCR and were therefore excluded. All samples were analysed in duplicate reactions for the presence of species C adenovirus DNA using hexon-specific nPCR-2 (Garnett *et al*, 2009). Only nine of the 727 samples yielded a positive result in at least one PCR reaction. These nine samples were re-tested using both nPCR-2 and nPCR-1, which detected a different region of the hexon gene (Garnett *et al*, 2009). Two of the nine samples were confirmed to contain adenoviral DNA by their yielding at least one positive result in each pair of reactions performed with both hexon-specific PCR assays. The other seven samples failed to yield a positive reaction with either PCR assay and were considered negative. Both positive samples were derived from ALL donor Guthrie cards. Both positive samples were confirmed to contain Ad2 DNA by sequencing the product of nPCR-1 (Garnett *et al*, 2002, 2009).

DISCUSSION

The reasons for the discrepancy between this investigation and our previous preliminary study are unclear. Quantitative analysis for a single-copy cellular gene indicated that the DNA present in Guthrie card extractions in this study was significantly more concentrated than that used in the preliminary report (data not shown). Controls analysed in the previous study eliminated the likelihood of PCR procedural contamination. Nonetheless, levels of adenovirus DNA measured by quantitative real-time PCR showed very low levels of viral DNA in the positive samples. We are unable to exclude the possibility of random environmental contamination in the previous study, although no negative controls were found to contain adenoviral DNA.

The low fraction of Guthrie cards positive for adenoviral DNA is in sharp contrast with studies on amniotic fluid. Of 1187 unique amniocentesis samples analysed in four separate studies of

sonographically normal pregnancies, more than 1 in 20 (5.4%) contained adenoviral DNA (Van den Veyver *et al*, 1998; Wenstrom *et al*, 1998; Baschat *et al*, 2003; Reddy *et al*, 2005). Remarkably, subsequent studies have detected no increased morbidity in infants following prenatal infection with adenovirus, as determined by PCR of amniocentesis samples (Miller *et al*, 2009), suggesting that prenatal infection, similar to postnatal infection, with species C adenoviruses is largely asymptomatic. Given a prenatal infection rate of 5%, our Guthrie card analysis seriously underestimates the frequency of fetal adenovirus infections, which is consistent with the biology of this pathogen. These results lead us to conclude that adenovirus DNA is not readily detected in the archived neonatal blood spots, irrespective of whether the subject subsequently develops leukaemia. Although the findings reported here fail to support our previously reported preliminary results (Gustafsson *et al*, 2007), these findings agree with those reported subsequently (Vasconcelos *et al*, 2008).

Adenovirus is not commonly found in peripheral blood, except during a fulminant infection (Flomenberg *et al*, 1997; Perlman *et al*, 2007). By contrast, a large majority of lymphocyte samples isolated from the adenoids or tonsils of normal children undergoing tonsillectomy or adenoidectomy contain adenovirus DNA (Garnett *et al*, 2002, 2009). Thus, after acute infection, adenovirus DNA may be restricted to mucosal lymphoid compartments in the infected neonate, as well as in the latently infected child, which may explain our inability to detect this virus in a high fraction of Guthrie cards containing peripheral blood.

Although these findings show no association between the development of leukaemia and adenovirus DNA in neonatal blood, this pathogen remains an intriguing candidate for a role in the aetiology of leukaemia. Species C adenovirus oncoproteins that disable the cellular DNA repair machinery (Weitzman and Ornelles, 2005) also have the power to transform cells by a 'hit and run' mechanism (Nevels *et al*, 2001; Endter and Dobner, 2004), making species C adenovirus a candidate for promoting the initial genetic lesion leading to leukaemia. Adenovirus may also have a role in the second hit postulated in the delayed infection model of leukaemia (Greaves, 2006). The fraction of children undergoing tonsillectomies with adenovirus DNA in their mucosal lymphocytes peaks between 2 to 5 years (see Figure 1 of Garnett *et al*, 2009), which coincides with the peak presentation of childhood ALL (Greaves, 2006).

ACKNOWLEDGEMENTS

We thank Marianne Alverstrand and Peter Castenbäck for their invaluable technical assistance. This work was supported by the Mary Béve Foundation for Paediatric Cancer Research, The Swedish Children Cancer Foundation, The Samariten Foundation, The Fernström foundation, a Government Public Health Grant (ALF), and by US PHS Grant CA127621.

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