

Short Communication

Antibody Responses to Simian Virus 40 T Antigen: A Case-Control Study of Non-Hodgkin Lymphoma

Eric A. Engels,¹ Jinbo Chen,¹ Patricia Hartge,¹ James R. Cerhan,² Scott Davis,³ Richard K. Severson,⁴ Wendy Cozen,⁵ and Raphael P. Viscidi⁶¹Division of Cancer Epidemiology and Genetics, National Cancer Institute, Department of Health and Human Services, Rockville, Maryland;²Mayo Clinic College of Medicine, Rochester, Minnesota; ³Fred Hutchinson Cancer Research Center, Seattle, Washington;⁴Karmanos Cancer Institute, Wayne State University, Detroit, Michigan; ⁵University of Southern California,Los Angeles, California; and ⁶Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

A possible role for SV40, a macaque polyomavirus, in non-Hodgkin lymphoma (NHL) in humans was raised recently by the reported detection of SV40 DNA in tumor tissue. Animals with SV40-induced tumors frequently produce high-level antibodies against T antigen, the SV40 oncoprotein. In this study, we assessed whether SV40 T antibody measured in humans supported a relationship between SV40 and NHL. Subjects were sampled from a U.S. population-based case-control study of NHL, according to presence of antibodies against capsids of SV40 and BK, a related human polyomavirus ($n = 85$ cases, $n = 95$ controls). T antibody was measured by enzyme immunoassay. We also evaluated serum specimens from SV40-infected and SV40-uninfected macaques ($n = 19$ and $n = 8$, respectively), SV40-uninfected hamsters ($n = 5$), and hamsters with SV40-induced tumors ($n = 10$). Hamsters with SV40-induced tumors all produced

robust SV40 T antibody [median absorbance, 0.99], whereas SV40-uninfected hamsters and macaques had much lower levels (median absorbance, 0.05 and 0.04, respectively). NHL cases, controls, and SV40-infected macaques resembled these latter two groups, generally showing only low-level T antibody (median absorbance, 0.03, 0.04, and 0.04, respectively). Overall, only five cases (6%) and five controls (5%) had T antibody responses classified as seropositive (odds ratio, 1.2; 95% confidence interval, 0.3–4.6). Interestingly, all 10 humans with T antibody responses also showed antibody responses to BK capsid. We found no association between the presence of T antibody and NHL, arguing against SV40 as a cause of NHL. Infrequent and low-level T antibody responses among humans could represent cross-reactivity to BK virus T antigen. (Cancer Epidemiol Biomarkers Prev 2005;14(2):521–4)

Introduction

The etiology of non-Hodgkin lymphoma (NHL) is largely unknown. Simian Virus 40, a macaque polyomavirus, can cause leukemia and lymphoma in laboratory rodents (1). Two research groups recently described the detection of SV40 DNA in tumor tissue from ~40% of U.S. NHL patients (2, 3). Some individuals might have acquired SV40 infection through receipt of poliovirus vaccines from 1955 to 1962, because SV40-contaminated poliovirus vaccines were used widely during this period (4). However, other groups subsequently failed to detect SV40 DNA in NHL tumors (5, 6), leaving the association uncertain. Similar difficulties have been encountered in studies of other tumors hypothesized to be related to SV40 (e.g., mesothelioma and brain tumors; ref. 4).

We recently conducted a U.S. population-based case-control study of SV40 infection and NHL (7). We tested subjects for antibodies to SV40 capsid using a virus-like particle (VLP) enzyme immunoassay (EIA; ref. 8). VLPs (empty capsids formed by spontaneous self-assembly of VP1, the major viral capsid protein) resemble native virions structurally and display B lymphocyte epitopes. In our study (7), only 7% to 10% of cases and 10% to 11% of controls exhibited antibody to SV40 capsid, and the responses were rather weak. In contrast, most individuals had measurable

antibody responses to capsids of BK and JC viruses, the polyomaviruses commonly infecting humans. Indeed, based on competitive inhibition experiments, we concluded that the low-level SV40 antibodies in our subjects mostly represented cross-reactive responses against BK or JC virus. These results, and similar results from Spain (9), point to a lack of frequent SV40 infection in NHL patients and in the general population (10).

SV40 transformation and immortalization of cell lines is mediated by T antigen, a viral replication protein that inactivates cellular tumor suppressor proteins p53 and pRb. In animals with SV40-induced cancer, SV40 T antigen is expressed within tumor cells (1, 11–13). As a result, these animals frequently produce high titers of antibody directed against T antigen (i.e., “T antibody”; ref. 11). In contrast, SV40-infected animals without tumors show only infrequent and weaker T antibody responses (11).

We hypothesized that if SV40 causes NHL in humans, antibody against SV40 T antigen should be detectable. This should be true even if SV40 replicates only inefficiently in humans, conceivably leading to weak antibody responses against viral capsid. In the present analysis, we therefore extended our antibody testing of U.S. NHL cases and controls by measuring SV40 T antibody. Results in humans were compared with those in SV40-infected and SV40-uninfected animals, including hamsters with SV40-induced tumors.

Materials and Methods

The National Cancer Institute-Surveillance, Epidemiology, and End Results case-control study has previously been described

Received 6/15/04; revised 9/9/04; accepted 9/21/04.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Eric A. Engels, Viral Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Department of Health and Human Services, 6120 Executive Boulevard, EPS 8010, Rockville, MD 20892.
E-mail: engelse@exchange.nih.gov

Copyright © 2005 American Association for Cancer Research.

(7). Briefly, HIV-uninfected subjects were enrolled between 1998 and 2000 from four U.S. areas: Iowa, and metropolitan Detroit, Los Angeles, and Seattle. Eligible cases with incident NHL (ages 20-74 years) were identified prospectively from the Surveillance, Epidemiology, and End Results registries. Eligible controls were identified using random digit dialing (ages 20-64 years) and Medicare eligibility files (65-74 years), sampled in strata defined by age, sex, race, and study area. The study was approved by institutional review boards at the National Cancer Institute and participating sites.

For the present analysis, we tested whether the frequency of measurable antibody against SV40 T antigen differed among cases and controls. To examine this question efficiently, we measured T antibodies only on a subset of subjects, sampled randomly from strata defined by SV40 VLP serostatus. Specifically, we included all SV40 VLP seropositive subjects and a random sample of SV40 VLP seronegative subjects, stratified by case-control status and BK VLP serostatus (Table 1). It would have been possible to sample subjects completely at random for T antibody testing. However, under the hypothesis that SV40 T antibody responses would be more frequent in SV40 VLP seropositive than in seronegative subjects, the enriched sampling of SV40 VLP seropositive subjects under this two-phase design has greater statistical power to detect an association between T antibody and NHL status (14). We also included sera from 19 SV40 VLP seropositive macaques and 8 SV40 VLP seronegative macaques. Finally, we simultaneously tested specimens from hamsters with tumors induced by s.c. inoculation of SV40-transformed cells (10 unique pooled serum specimens, each created from the sera of three to five animals) and five specimens from SV40-uninfected hamsters (including one pooled serum specimen).

EIA microtiter plates were sensitized with full-length, purified SV40 large T antigen (250 ng/well), produced in *sf9* insect cells from a recombinant baculovirus construct (15). Using standard EIA methods, coated plates were incubated with serum samples (diluted 1:100), washed, incubated with peroxidase-conjugated goat antibodies against human immunoglobulin G (for human and macaque samples) or hamster immunoglobulin G, and treated with 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) hydrogen peroxide solution to colorimetrically measure T antibodies. Samples were assayed in duplicate, and we utilized the mean absorbance measurement for each sample. Results on 17 human samples are missing due to low serum volume.

As an assay cutoff, we chose three standard deviations above the mean absorbance measurement for SV40 VLP seronegative macaques (i.e., $0.04 + 3 \times 0.02 = 0.10$). Assuming that assay results are normally distributed, this cutoff would classify 99.9% of SV40 VLP seronegative (presumably SV40-uninfected)

macaques as T antibody seronegative (i.e., high specificity). Although it is unlikely that even weaker antibody responses measured could be considered seropositive, in sensitivity analyses, we evaluated lower cutoffs.

We fitted a logistic regression model to assess whether SV40 T antibody status was related to case-control status. We used methods developed by Breslow and Holubkov (16) to adjust for two-stage sampling. The odds ratio was additionally adjusted for sex, race, age, and study site.

Results

Cases ($n = 85$) and controls ($n = 95$) tested for T antibody in the present analysis resembled the larger groups tested previously for SV40 capsid antibody (7). Among subjects in this analysis, 65% of cases and 51% of controls were male ($P = 0.05$). Cases and controls resembled each other with respect to age (mean 58 versus 59 years, respectively; $P = 0.69$) and with respect to race and study site (data not shown). NHLs were classified as follicular (25%), diffuse large B cell (26%), T cell (7%), or other/unknown (42%) subtypes. By site, 60% of NHLs were nodal and 40% were extranodal.

T antibody results are shown in Fig. 1. Hamsters with SV40-induced tumors all had readily measurable SV40 T antibody (median absorbance, 0.99; range, 0.73-1.48), whereas SV40-uninfected hamsters and SV40 VLP seronegative macaques uniformly gave substantially lower results (median absorbance, 0.05; range, 0.03-0.07; and median absorbance, 0.04; range, 0.01-0.08, respectively). NHL cases, controls, and SV40 VLP seropositive macaques resembled these latter two groups, generally showing very low measurable SV40 T antibody (median absorbance, 0.03, 0.04, and 0.04, respectively; Fig. 1).

Overall, five cases and five controls (6% versus 5%; Table 1) and two SV40-infected macaques (11%) had T antibody responses classified as seropositive. T antibody responses in humans and macaques were all weaker than seen in hamsters with tumors (Fig. 1). SV40 T antibody seropositivity was unrelated to NHL (adjusted odds ratio, 1.2; 95% confidence interval, 0.3-4.6). SV40 T antibody was detected in cases with varied histologic types of NHL and sites of disease (Table 2). Two T antibody seropositive cases had cutaneous T cell NHL, but only one of these also had SV40 VLP antibody (Table 1); four other T cell NHLs were T antibody seronegative.

Among our controls, we explored whether SV40 T antibody was associated with VLP antibody results, although small numbers prevented strong conclusions (Table 1). SV40 T antibody was present in 6% of SV40 VLP seropositive controls and 3% of SV40 VLP seronegative controls. Interestingly, SV40 T antibody was detected only in BK VLP seropositive controls (7%), a finding that was mirrored in the cases (8%). T antibody

Table 1. Case-control subjects sampled for SV40 T antibody measurement and results of testing

SV40 VLP serostatus	Case-control status	BK VLP serostatus	Subjects in NCI-SEER case-control study	Evaluated for T antibody, <i>n</i> (%) [*]	Positive for T antibody, <i>n</i> (%) [†]
+	Case	+	49	45 (92)	3 (7)
+	Case	-	3	3 (100)	0 (0)
+	Control	+	58	55 (95)	4 (7)
+	Control	-	7	7 (100)	0 (0)
-	Case	+	419	19 (5)	2 (11)
-	Case	-	253	18 (7)	0 (0)
-	Control	+	343	16 (5)	1 (6)
-	Control	-	214	17 (8)	0 (0)
Any	Case	Any	724	85 (12)	5 (6)
Any	Control	Any	622	95 (15)	5 (5)

Note: Abbreviations: VLP, virus-like particle; NCI-SEER, National Cancer Institute-Surveillance, Epidemiology, and End Results.

^{*}Subjects evaluated for T antibody, expressed as a percentage of all such subjects in National Cancer Institute-Surveillance, Epidemiology, and End Results case-control study. The number evaluated excludes 17 subjects for whom low serum volume precluded T antibody measurement.

[†]Subjects positive for T antibody, expressed as a percentage of such subjects evaluated for T antibody.

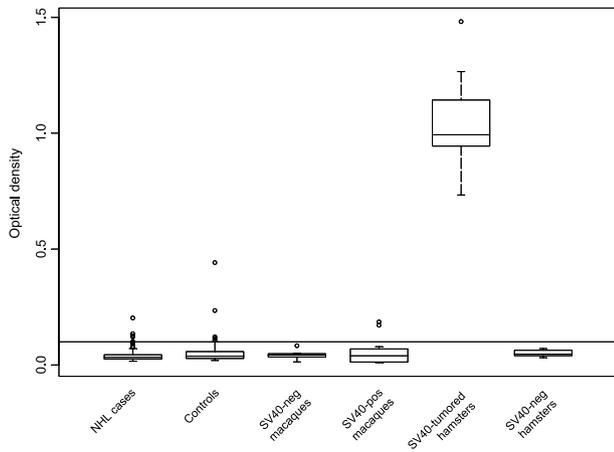


Figure 1. SV40 T antibody results, based on an EIA detecting antibodies to recombinant SV40 T antigen. Humans and macaques were sampled according to their antibody status on VLP EIA (see Materials and Methods). *Box plots*, absorbance results for NHL cases ($n = 85$) and controls ($n = 95$), SV40 VLP seronegative macaques ($n = 8$), SV40 VLP seropositive macaques ($n = 19$), hamsters with SV40-induced tumors ($n = 10$), and SV40-uninfected hamsters ($n = 5$). *Horizontal line*, median absorbance value; *box*, interquartile range; *points*, outliers. *Long horizontal line*, assay cutoff defining a seropositive result (0.10 absorbance units).

was detected in 3 of 69 (4%) JC VLP seropositive controls and 2 of 26 (8%) JC VLP seronegative controls.

Lowering the cutoff for the T antibody EIA classified more subjects as seropositive, but fewer cases than controls were classified as such. With a cutoff of 0.08 absorbance units, 8% of cases and 13% of controls were considered T antibody seropositive. With a cutoff of 0.06 absorbance units, 18% of cases and 24% of controls were considered T antibody seropositive.

Discussion

We observed a lack of antibody to SV40 T antigen among humans, including individuals with NHL. In animals with SV40-induced tumors, T antigen is present within each tumor cell (1, 12, 13). T antigen expression in tumors leads to a robust antibody response against this oncoprotein, as we showed for hamsters with SV40-induced tumors and as shown previously (11). Even when we lowered our assay's cutoff to increase its sensitivity, T antibody seroprevalence in NHL cases was not higher than in controls.

To our knowledge, only one prior investigation systematically evaluated cancer patients for SV40 T antibody. Shah (17) studied 975 individuals from northern India, where contact with indigenous SV40-infected rhesus might lead to SV40 infections in humans. The study included 26 patients with malignancy of the lymphatic system, 16 with "reticulum cell sarcoma" (now classified as NHL), and 14 with leukemia. Only 5% of cancer patients overall (4-7% of patients with hematologic malignancies) showed SV40 neutralizing antibody, generally at low titer. Notably, no patient had SV40 T antibody as measured by an immunofluorescence assay.

In contrast, investigations that have used sensitive PCR methods to test for SV40 DNA in NHL tumor tissue have produced highly variable results. Studies from the U.S., Europe, and Japan have reported detection rates ranging from 0% to 43% (2, 3, 5, 6, 18-21). Geographic differences across study populations might be relevant, but there were also differences in methods among laboratories, such as the choice of PCR primers and reaction conditions. Some PCR-based studies reporting positive results suffered from methodologic problems, including a lack of masked evaluation of specimens (2, 3, 5, 18-21), lack of appropriate negative controls (5, 18-21), and the amplification of SV40 sequences from negative control tissues (2). Furthermore, the amount of SV40 DNA in PCR-positive specimens seemed quite low (19, 21). Because SV40 is present within each cell in animal lymphomas (1), the reported detection of SV40 DNA at extremely low copy numbers in human tumors could represent a laboratory artifact, e.g., PCR contamination.

Furthermore, there is no epidemiologic evidence linking SV40 to NHL in humans. Between 1955 and 1962, millions of individuals in the U.S. and Europe were exposed to SV40 via contaminated poliovirus vaccines (4). Nonetheless, individuals exposed to SV40-contaminated poliovirus vaccines do not have an elevated NHL risk (22). SV40 infection routes other than vaccination have not been shown. Unlike BK and JC viruses, SV40 does not seem to be shed in human urine nor has it been detected in sewage (23, 24), arguing against person-to-person transmission and frequent infection.

We found SV40 T antibody to be a sensitive and specific marker for SV40-induced malignancy. All five evaluated sera from animals with SV40-related tumors manifested robust T antibody levels using our EIA. In animals, the frequency of T antibody detection and antibody titer increase with tumor size (11, 13). Because NHL is a systemic disease, generally with a large tumor burden at the time of clinical presentation, we would expect our test to have high sensitivity for detecting SV40-related NHLs in humans. Conversely, because only 5% of human controls in our study had measurable T antibody responses, the EIA likely has at least 95% specificity. Interestingly, all T antibody seropositive humans were BK VLP seropositive. This finding could indicate that some low-level SV40 T antibody responses

Table 2. Subjects with positive SV40 T antibody responses

Case-control status	Sex, age (years)	Birth year	NHL		VLP serostatus		
			Histology	Site	SV40	BK	JC
Cases	Male, 46	1953	DLBC	Lymph node	+	+	-
	Male, 49	1950	T cell	Skin	-	+	+
	Male, 66	1932	T cell	Skin	+	+	+
	Female, 70	1928	Follicular	Intestine	-	+	-
	Male, 72	1926	Not specified	Intestine	+	+	+
Controls	Male, 45	1953	n/a	n/a	+	+	+
	Female, 63	1936	n/a	n/a	+	+	+
	Male, 68	1931	n/a	n/a	+	+	-
	Female, 70	1929	n/a	n/a	-	+	-
	Female, 70	1928	n/a	n/a	+	+	+

Note: Abbreviations: VLP, virus-like particle; DLBC, diffuse large B cell; n/a, not applicable.

in humans actually represent cross-reacting antibody responses to BK virus T antigen (25).

A limitation of our study was that we did not have archived tumor tissue available, which might have allowed us to compare the SV40 DNA status of tumors with these antibody results. However, methods for SV40 DNA detection are not standardized (4) and, as noted above, issues remain regarding the biological interpretation of PCR results. A further limitation is our small sample size with associated wide confidence limits, although our overall results provide little justification for evaluating a larger collection of NHL samples.

In conclusion, the present study provides evidence that SV40 is not a major cause of NHL in the U.S. Our subjects only rarely had antibody to SV40 T antigen. The frequency of these antibodies did not differ between NHL cases and controls, and the low antibody levels are most consistent with cross-reactivity to BK virus T antigen. Additional serologic studies of patients with NHL and other tumors possibly related to SV40 will be valuable (4).

Acknowledgments

We thank Ronald Kennedy and Michael Shearer (Texas Tech University Health Science Center, Lubbock, TX) for providing recombinant SV40 T antigen, and Barbara Clayman (Johns Hopkins University) for conducting the serology testing. We gratefully acknowledge the contributions of the study participants and the staff at the SEER centers of Detroit, Iowa, Los Angeles, and Seattle. Supports for this study included the following contracts with the National Cancer Institute: N01-PC-67010, N01-PC-67008, N02-PC-71105, N01-PC-67009, and N01-PC-65064.

References

1. Diamandopoulos GT. Leukemia, lymphoma, and osteosarcoma induced in the Syrian golden hamster by simian virus 40. *Science* 1972;176:173–5.
2. Shivapurkar N, Harada K, Reddy J, et al. Presence of simian virus 40 DNA sequences in human lymphomas. *Lancet* 2002;359:851–2.
3. Vilchez RA, Madden CR, Kozinetz CA, et al. Association between simian virus 40 and non-Hodgkin lymphoma. *Lancet* 2002;359:817–23.
4. Anonymous. Immunization safety review. SV40 contamination of polio vaccine and cancer. Washington, DC: National Academy Press; 2003.
5. Capello D, Rossi D, Gaudino G, Carbone A, Gaidano G. Simian virus 40 infection in lymphoproliferative disorders. *Lancet* 2003;361:88–9.

6. MacKenzie J, Wilson KS, Perry J, Gallagher A, Jarrett RF. Association between simian virus 40 DNA and lymphoma in the United Kingdom. *J Natl Cancer Inst* 2003;95:1001–3.
7. Engels EA, Viscidi RP, Galloway DA, et al. Case-control study of simian virus 40 and non-Hodgkin lymphoma in the United States. *J Natl Cancer Inst* 2004;96:1368–74.
8. Viscidi RP, Rollison DEM, Viscidi E, et al. Serological cross-reactivities between antibodies to simian virus 40, BK virus, and JC virus assessed by virus-like-particle-based enzyme immunoassays. *Clin Diagn Lab Immunol* 2003;10:278–85.
9. de Sanjose S, Shah KV, Domingo-Domenech E, et al. Lack of serological evidence for an association between simian virus 40 and lymphoma. *Int J Cancer* 2003;104:522–4.
10. Shah KV, Galloway DA, Knowles WA, Viscidi RP. Simian virus 40 (SV40) and human cancer: a review of the serological data. *Rev Med Virol* 2004;14:231–9.
11. Black PH, Rowe WP, Turner HC, Huebner RJ. A specific complement-fixing antigen present in SV40 tumor and transformed cells. *Proc Natl Acad Sci U S A* 1963;50:1148–56.
12. Pope JH, Rowe WP. Detection of specific antigen in SV40-transformed cells by immunofluorescence. *J Exp Med* 1964;120:121–8.
13. Rapp F, Butel JS, Melnick JL. Virus-induced intranuclear antigen in cells transformed by papovavirus SV40. *Proc Soc Exp Biol Med* 1964;116:1131–5.
14. White JE. A two-stage design for the study of the relationship between a rare exposure and a rare disease. *Am J Epidemiol* 1982;115:119–28.
15. Lanford RE. Expression of simian virus 40 T antigen in insect cells using a baculovirus expression vector. *Virology* 1988;167:72–81.
16. Breslow NE, Holubkov R. Maximum likelihood estimation of logistic regression parameters under two-phase, outcome-dependent sampling. *J R Stat Soc (Series B, Methodological)* 1997;59:447–61.
17. Shah KV. Investigation of human malignant tumors in India for simian virus 40 etiology. *J Natl Cancer Inst* 1969;42:139–45.
18. Rizzo P, Carbone M, Fisher SG, et al. Simian virus 40 is present in most United States human mesotheliomas, but it is rarely present in non-Hodgkin's lymphoma. *Chest* 1999;116:470–3S.
19. David H, Mendoza S, Konishi T, Miller CW. Simian virus 40 is present in human lymphomas and normal blood. *Cancer Lett* 2001;162:57–64.
20. Nakatsuka S, Liu A, Dong Z, et al. Simian virus 40 sequences in malignant lymphomas in Japan. *Cancer Res* 2003;63:7606–8.
21. Martini F, Dolcetti R, Gloghini A, et al. Simian-virus-40 footprints in human lymphoproliferative disorders of HIV– and HIV+ patients. *Int J Cancer* 1998;78:669–74.
22. Engels EA, Katki HA, Nielsen NM, et al. Cancer incidence in Denmark following exposure to poliovirus vaccine contaminated with simian virus 40. *J Natl Cancer Inst* 2003;95:532–9.
23. Shah KV, Daniel RW, Strickler HD, Goedert JJ. Investigation of human urine for genomic sequences of the primate polyomaviruses simian virus 40, BK virus, and JC virus. *J Infect Dis* 1997;176:1618–21.
24. Bofill-Mas S, Pina S, Girones R. Documenting the epidemiologic patterns of polyomaviruses in human populations by studying their presence in urban sewage. *Appl Environ Microbiol* 2000;66:238–45.
25. Shah KV, Daniel RW, Murphy G. Antibodies reacting to simian virus 40 T antigen in human sera. *J Natl Cancer Inst* 1973;51:687–90.