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研究報告の概要

ヒトパピローマウイルス（HPV）は上皮細胞親和性のウイルスであり、その生活環には扁平上皮の分化という環境を必要とする。皮膚の剥脱や性交により HPV に感染すると、良性の疣やときには癌が発現する。これまで、血液中から検出される HPV の DNA は、転移癌細胞に由来すると考えられてきた。本研究では、1987 年から 1996 年に米国のヒト免疫不全ウイルス（HIV）感染小児科患者 57 例から採取し凍結保存した末梢血単核細胞（PBMC）、および 2002 年から 2003 年に健康な供血者 19 例から採取した新鮮 PBMC について HPV DNA を検査した。患児 8 例と供血者 3 例が、HPV 16 型ゲノムの 2 つのサブグループに関してほぼ陽性を示した。11 例すべての PBMC サンプルから検出された HPV ゲノムは、DNA コピー数は少ないものの、エピソーム型として存在していた。患児 8 例のうち 7 例（3 例は血友病患者）は輸血により、1 例は垂直感染により HIV に感染したもので、垂直感染による 1 例もサンプル採取前に輸血が行われていた。本研究データは、PBMC が HPV 担体であり、血液を通じてウイルスを伝播する可能性があることを示唆している。

報告企業の意見	今後の対応
PBMCがHPV担体であり、血液を通じてウイルスを伝播する可能性があることを示唆している報告である。 血漿分画製剤からのHPV伝播の事例は報告されていない。また、万一原料血漿にHPVが混入したとしても、HPVと類似した特徴を有しているCPVのウイルスバリデーション試験成績から、献血ヴェノグロブリン-IHヨシトミ及びヴェノグロブリン-IHの製造工程において十分に不活化・除去されると考えている。グロブリン-Wfについては、CPVのウイルスバリデーション試験成績からは、製造工程において不活化・除去が十分であるとは説明困難であるため、万一HPVの原血漿への混入が判明した場合にはその事実を総合機構及び厚生労働省に報告する。	HPV の原血漿への混入が判明した場合にはその事実を総合機構及び厚生労働省に報告する。

使用上の注意記載状況・その他参考事項等

代表として献血ヴェノグロブリン-IH ヨシトミの記載を示す。
2. 重要な基本的注意
(1)本剤の原材料となる献血者の血液については、HBs 抗原、抗 HCV 抗体、抗 HIV-1 抗体、抗 HIV-2 抗体、抗 HTLV-I 抗体陰性で、かつ ALT（GPT）値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV 及び HCV について核酸増幅検査（NAT）を実施し、適合した血漿を本剤の製造に使用しているが、当該 NAT の検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した血漿を原料として、Cohn の低温エタノール分画で得た画分からポリエチレングリコール 4000 処理、DEAE セファデックス処理等により人免疫グロブリンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において 60℃、10 時間の液状加熱処理及び濾過膜処理（ナノフィルトレーション）を施しているが、投与に際しては、次の点に十分注意すること。

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Could Human Papillomaviruses Be Spread through Blood?

Sohrab Bodaghi,[1] Lauren V. Wood,[1] Gregg Roby,[1] Celia Ryder,[1] Seth M. Steinberg,[2] and Zhi-Ming Zheng[1]*

HIV and AIDS Malignancy Branch[1] and Biostatistics and Data Management Section,[2] Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

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The human papillomaviruses (HPVs) are epitheliotropic viruses that require the environment of a differentiating squamous epithelium for their life cycle. HPV infection through abrasion of the skin or sexual intercourse causes benign warts and sometimes cancer. HPV DNA detected in the blood has been interpreted as having originated from metastasized cancer cells. The present study examined HPV DNA in banked, frozen peripheral blood mononuclear cells (PBMCs) from 57 U.S. human immunodeficiency virus (HIV)-infected pediatric patients collected between 1987 and 1996 and in fresh PBMCs from 19 healthy blood donors collected in 2002 to 2003. Eight patients and three blood donors were positive mostly for two subgroups of the HPV type 16 genome. The HPV genome detected in all 11 PBMC samples existed as an episomal form, albeit at a low DNA copy number. Among the eight patients, seven acquired HIV from transfusion (three associated with hemophilia) and one acquired HIV through vertical transmission; this patient also had received a transfusion before sampling. Our data suggest that PBMCs may be HPV carriers and might spread the virus through blood.

Sexual transmission of and infection with human papillomaviruses (HPVs) are widely recognized as a cause of anogenital warts and cervical cancer. The infection through abrasion of the skin or sexual intercourse is initiated when a viral particle gains entry into a basal epithelial cell. While all cells of a wart contain the viral genome, viral gene expression and multiplication occur exclusively in the nuclei of the infected cells and are tightly linked to the state of differentiation of the cells. In basal and parabasal cells, viral DNA replicates at a low level as an episome and only early genes are transcribed. Extensive viral DNA multiplication and transcription of all viral genes as well as capsid formation occur only in the most superficial layers of the epithelium (14). It has been widely accepted that HPVs are not disseminated to other sites by blood, i.e., there is no viremic phase in the course of HPV infection. However, successful transmission of bovine papillomavirus type 2 from peripheral blood (35) raises the possibility that HPVs might in some circumstances be spread via a hematogenous route. In addition, HPV DNA can be detected in the peripheral blood mononuclear cells (PBMCs) (29), sera (22), or plasma (9) of patients with cervical cancer or HPV-associated head and neck squamous cell carcinoma (5). It should therefore be considered whether PBMCs might serve as a carrier of HPV during the course of HPV infection.

Women with human immunodeficiency virus (HIV) infection have a high prevalence of cervical HPV infection and cervical cancer (10, 36). Although both HIV and HPV are sexually transmitted and this could partly account for the higher prevalence of HPV infection in HIV-positive patients, HIV-associated immunosuppression might contribute to reactivation of preexisting HPV infection and predispose patients to progression to high-grade squamous intraepithelial lesions (1, 25). Also, HIV infection of $CD4^+$ cells might hypothetically reactivate HPV within the PBMCs if the HPV genome resides in the cells.

In this report, we have examined HPV DNA in PBMCs obtained from HIV-infected pediatric patients and healthy blood donors. Our data document that the HPV genome is associated with PBMCs and hence could potentially be spread through blood transfusion.

MATERIALS AND METHODS

PBMC acquisition and DNA extraction. To determine the presence of HPV infection in PBMCs, a total of 76 banked, frozen PBMC samples obtained between 1987 and 1996 from 57 U.S. pediatric patients with vertical or transfusion-acquired HIV infection, with a median age of 13.2 years (Table 1), enrolled in National Cancer Institute (NCI) Institutional Review Board-approved protocols, were analyzed. All clinical blood specimens obtained from pediatric patients were obtained by nurses or phlebotomists wearing gloves. PBMCs were isolated from clinical specimens by the standard Ficoll-Hypaque gradient separation technique and cryopreserved in a vapor-phase liquid nitrogen storage freezer. A total of 24 PBMC samples from 19 healthy blood donors without clinical complaints at the time of donation were also collected from the NIH Clinical Center blood bank over a period of 6 months in 2002 to 2003 and were isolated by a centrifugal elutriation technique performed by the Cell Processing Section of the NIH Clinical Center blood bank. For HIV-positive samples, all PBMC samples, each at $>2 \times 10^6$, were randomly coded and blinded, with random duplications as internal controls. DNA samples were extracted directly from PBMCs by brief centrifugation and homogenized in 1 ml of DNAzol (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. The isolated DNA was dissolved in ~500 μl of 8 mM NaOH and adjusted to pH 7.0 with 1 M HEPES. Various precautions were taken to minimize sample-to-sample cross-contamination, including limiting HIV-PBMC sample processing and DNA extraction to a maximum of 10 samples per day.

HPV DNA detection and sequencing. Detection of HPV L1 and HPV type 16 (HPV16) E2 and E6 genes either from randomly coded and blinded PBMC DNA samples or from blood donor PBMC DNA samples was performed by nested PCR as described previously (3). After the PCR products for L1 were sequenced and an HPV type was confirmed, two sets of HPV type-specific E6 and E2 primers for nested PCR were further applied for HPV type-specific detection

* Corresponding author. Mailing address: HIV and AIDS Malignancy Branch, Center for Cancer Research, NCI/NIH, 10 Center Dr., Rm. 10 S255, MSC-1868, Bethesda, MD 20892-1868. Phone: (301) 594-1382. Fax: (301) 480-8250. E-mail: zhengt@exchange.nih.gov.

TABLE 1. Demographic characteristics and prevalence of HPV DNA in PBMCs of pediatric HIV patients and healthy blood donors

Characteristic	No. (%) of patients or donors[a]			P^b
	Total	HPV-pos	HPV-neg	
Pediatric HIV patients	57	8[c]	49	
Mode of HIV acquisition				
Transfusion	17	4 (23.5)	13 (76.5)	0.35*
Hemophilia	21	3 (14.3)	18 (85.7)	
Vertical	19	1 (5.3)	18 (94.7)	
CD4 count/mm^3				
Minimal (>500)	4	0	4	0.78**
Moderate (200–500)	10	2 (20)	8 (80)	
Severe (<200)	43	6 (14)	37 (86)	
Gender				
Male	40	5 (12.5)	35 (87.5)	0.68***
Female	17	3 (17.6)	14 (82.4)	
Age[d] (yr)				
≤13	27	3 (11.1)	24 (88.9)	0.38****
>13	30	5 (16.7)	25 (83.3)	
Healthy blood donors	19	3	16	
Gender				
Male	16	2 (12.5)	14 (87.5)	0.42***
Female	3	1 (33.3)	2 (66.7)	
Race				
White	11	0	11	0.06***
Black	8	3 (37.5)	5 (62.5)	
Age[d] (yr)				
20–35	8	3 (37.5)	5 (62.5)	0.08****
≥36	11	0	11 (100)	

[a] HPV-pos and HPV-neg, HPV positive and negative, respectively.

[b] Two-tailed P value for HPV positive versus HPV negative. *, by Mehta's version of Fisher's exact test for all three acquisition categories versus HPV positivity. P = 0.25 by Fisher's exact test for vertical versus transfusion/hemophilia acquired: odds ratio = 0.25 (95% exact confidence interval, 0.005 to 2.22). **, by exact Cochran-Armitage trend test. ***, by Fisher's exact test. ****, by Wilcoxon rank sum test.

[c] P = 1.00 for 8/57 versus 3/19 HPV positive in pediatric HIV patients versus healthy blood donors.

[d] Median ages, in years, with ranges in parentheses, were as follows: for total, HPV-positive, and HPV-negative pediatric HIV patients, 13.2 (2 to 29), 13.3 (10 to 18), and 13.0 (2 to 29), respectively; for total, HPV-positive, and HPV-negative healthy blood donors, 36 (23 to 71), 34 (23 to 35), and 40.5 (29 to 71), respectively.

and sequencing. By combining the detection for L1, E2, and E6 genes that cover the two ends and middle part of the virus genome, this strategy allowed us to analyze whether a full-length HPV genome existed in the PBMCs. Head-to-tail junctions of HPV genomes were further analyzed to determine the presence of an episomal HPV genome as detailed in Fig. 3. Two sets of HPV16-specific primers for nested PCR were used for the detection, including two forward primers, Pr7581 (5′-CACTGCTTGCCAACCATTCC-3′) and Pr7677 (5′-GCC AACGCCTTACATACCG-3′), and two backward primers, Pr128 (5′-GTCGC TCCTGTGGGTCCTG-3′) and Pr223 (5′-ACGTCGCAGTAACTGTTGC-3′).

Gel-purified PCR products with the expected sizes were used as DNA templates in cycle-sequencing reactions (BigDye Terminator cycle sequencing kit; Applied Biosystems, Foster City, CA) from two different directions. Sequencing reaction mixtures were purified using Centricep Spin columns (Princeton Separations, Adelphia, NJ) and were run in the Applied Biosystems model 377 sequencing apparatus. Sequence data compiled were analyzed using Sequencher sequence analysis software (Gene Codes Corp., Ann Arbor, MI).

Validation of PCRs. Each DNA sample was screened for the presence of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA by PCR amplification with a primer set as previously described (3). This primer set amplifies a 496-bp product and provides an indication of good DNA quality for each sample. For HPV detection, two water controls were also included for both first-run and nested PCR. If either of the two water controls yielded a false positive in the nested PCR, the whole set of PCRs and nested PCRs were restarted. A sample was deemed to be HPV positive if it yielded PCR products at least twice in a total of three repeats with the expected sizes for both L1 and E6 that could be further confirmed by DNA sequencing. This high stringency allowed us to exclude any possibility of laboratory cross-contamination in the nested PCR. Sample unblinding was then performed after completion of the detection and sequencing.

RESULTS

Presence of HPV genome in PBMCs of pediatric HIV patients. Of the 76 samples from 57 pediatric HIV patients, 10 samples (two duplicates from the same draw date) from eight patients were positive for HPV16 DNA (Fig. 1). Of the eight patients (14%) with HPV detected, seven had transfusion-acquired HIV (three associated with hemophilia) and one had vertical infection with a history of transfusion before sampling (Table 1). Although there was no significant difference in this small study in the rate of HPV DNA detection according to the patients' mode of HIV acquisition (P = 0.35) or in the overall ages of patients with or without HPV (P = 0.38), the data suggest that the PBMC HPV DNA in these patients might be acquired through blood products. Among those eight patients with PBMC HPV DNA, three were 13 years of age, two 11, one 14, one 17, and one 18 years of age when the blood samples

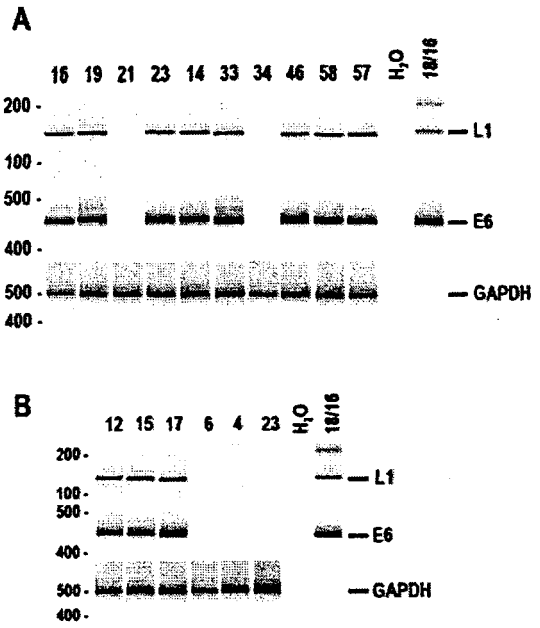

FIG. 1. Electrophoretic profiles of HPV L1 and HPV16 E6 DNA products amplified from banked PBMCs of pediatric HIV patients (A) and from PBMCs of healthy blood donors (B). HPV L1 and HPV16 E6 genes were detected, respectively, from purified total PBMC DNA by nested PCR with primer sets as described previously (3). PCR amplification was performed as described previously (3) with a primer set for the human GAPDH gene serving as a DNA quality control for each sample. Gel images are representatives of amplified products analyzed with an Agilent 2100 Bioanalyzer. Shown on the top of each panel are patient sample numbers, water controls, and HPV18 DNA (for L1) as well as HPV16 DNA (for E6) controls. Numbers at left of panels are molecular sizes in base pairs.

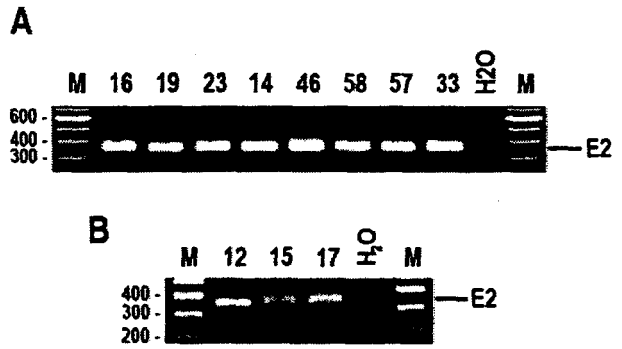

FIG. 2. Detection of intact HPV16 E2 gene in all of 11 HPV16-positive PBMCs. Total PBMC DNA was detected by nested PCR with HPV16 E2 primer sets as described previously (3). (A) Intact E2 in pediatric HIV patients with HPV-positive PBMCs. (B) Intact E2 in healthy blood donors with HPV-positive PBMCs. Shown on the top of each panel are patient (A) or donor (B) sample numbers and water controls. Lanes M, molecular size markers (sizes at left in base pairs).

were drawn. According to clinical history, all of the HPV-positive pediatric patients were sexually naive at that time.

Analysis of 19 duplicate samples from 13 patients with more than one sample tested showed that five patients had duplicate samples with the same results, with two duplicates positive and three duplicates negative for HPV DNA, indicating reliable HPV DNA detection. Other patients with samples from different time points, including samples from two patients positive for HPV DNA, were negative for HPV DNA at another draw date at least 5 months distant, implying a fluctuation of HPV DNA levels in HIV patients' PBMCs. All eight patients with PBMC HPV DNA had moderate (two patients) or severe (six patients) immune suppression (Table 1).

Detection of HPV genome in PBMCs of healthy blood donors. To further explore the possibility that blood transfusion could be a source of acquired PBMC HPV DNA, we obtained 24 PBMC samples from 19 healthy blood donors over a period of 6 months and examined them for HPV DNA using the same HPV DNA detection strategy as described above. Three donors (15.8%) were also positive for HPV16 DNA in their PBMCs (Table 1; Fig. 1). Interestingly, two donors with multiple samples at different time points were documented to have HPV16 DNA only once, again suggesting that detection of HPV16 DNA in PBMCs may be transient.

PBMCs carry an episomal HPV genome. To determine the physical status of HPV DNA detected in the PBMCs, the HPV16 E2 gene from all 11 HPV16 DNA-positive PBMCs was examined. In general, an intact E2 gene is disrupted upon HPV integration, thus distinguishing the episomal form of HPV DNA from the integrated form by detection of an intact E2 gene. Although it is indirect, amplification of the E2 region indicates the presence of episomal HPV DNA in the PBMCs; otherwise, it is assumed that the DNA has integrated (18, 39). Using this approach, we demonstrated that all 11 HPV16 DNA-positive PBMC specimens had the intact HPV16 E2 gene (Fig. 2), suggesting that the detected HPV genome in the PBMCs is episomal.

To further confirm the presence of episomal HPV16 DNA in the PBMCs, we detected the head-tail junctions of episomal HPV16 genomes in assuming that an episomal genome should be circular. We found that all of the 11 HPV16-positive PBMC DNA samples from pediatric HIV patients and healthy blood donors rendered an amplicon with correct sizes by nested PCR with two sets of primers, the forward primers covering the end of the HPV16 genome and the backward primers being positioned at the beginning of the genome (Fig. 3). All of the 11 amplicons were sequenced and showed a correct head-tail junction (. . .TAATACTAA-7906/1-ACTACAA. . . . .), demonstrating the existence of the circular (episomal) HPV16 genomes in the PBMCs.

Two subgroups of HPV genomes in PBMCs. Sequence analysis of all HPV16 E6 and E2 amplicons from PBMCs indicated that they were European variants and amplified mainly from two subgroups of the HPV16 genome that have not been reported in genital or cervical variants and are different from our laboratory strains (HPV16R, CaSki and SiHa) (Table 2), convincingly indicating that the detected HPV16 DNA in PBMCs was not a result of cross-contamination in our laboratory. One subgroup (five isolates) has an A-to-T change at the nucleotide (nt) 362 position in conjunction with a C-to-A change at the nt 3684 position, subsequently resulting in a missense mutation in the E6 (T87S) and E2 (T310K) proteins, respectively. The other subgroup (four isolates) has prototype HPV16R sequences in the corresponding positions, but three of them have a T-to-G change at the nt 350 position, leading to

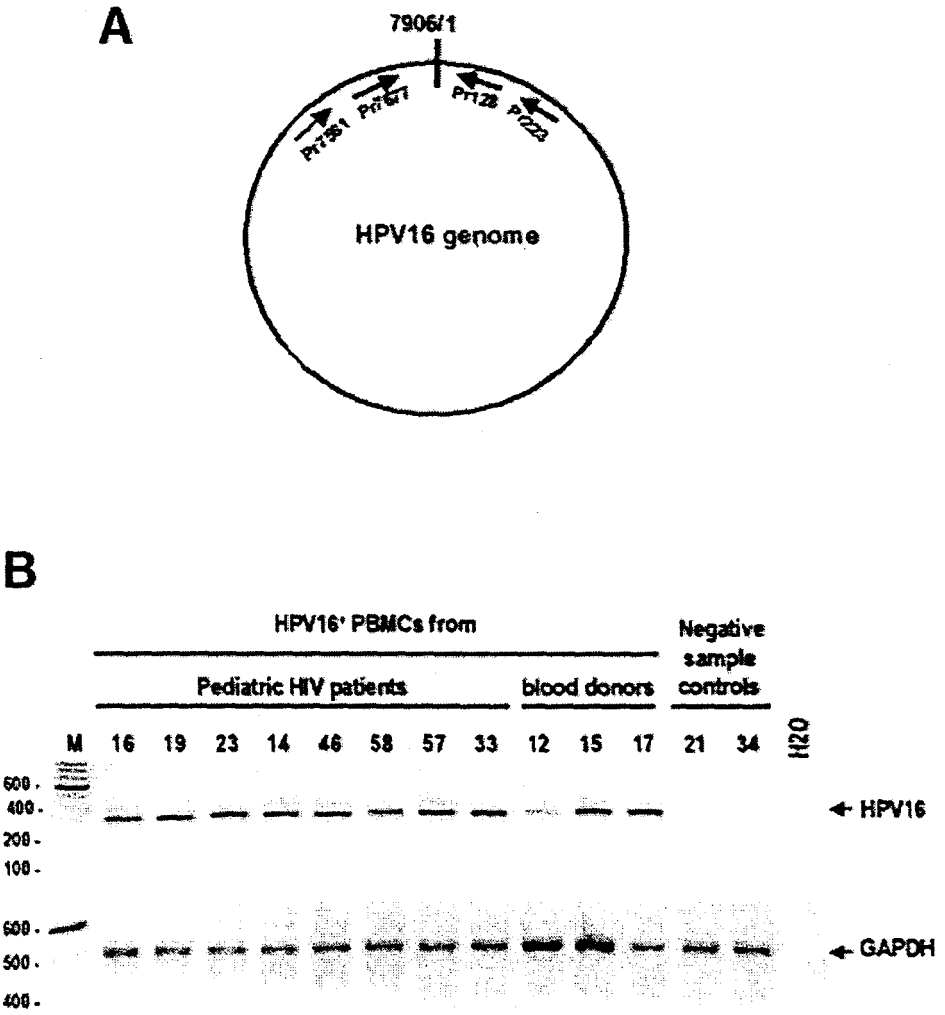

FIG. 3. Detection of head-to-tail junctions of episomal HPV16 genomes in HPV16-positive PBMCs from pediatric HIV patients and healthy blood donors. (A) Schematic diagram of circular, episomal HPV16 genome and relative positions of the PCR primers used in this study. Drawings are not to scale. (B) Head-to-tail junction products amplified from individual HPV16-positive PBMC DNA samples by the nested PCR in which the primers Pr7581 and Pr223 were used for the first PCR and the primers Pr7677 and Pr128 were used for the nested PCR. Shown on the top of the gel are patient or donor sample numbers. HPV16-negative PBMC DNA samples (21 and 34) in Fig. 1 were used as negative controls. See the description of GAPDH for DNA quality control in Fig. 1. Lane M, molecular size markers (sizes at left in base pairs).

an amino acid change (L83V) in the E6 protein. Two other isolates from pediatric HIV patients could not be grouped: one (patient 19) has the same nucleotide sequences at those positions as seen in CaSki HPV16, but the nucleotide sequence at the nt 350 position could not be determined as a T or G in multiple sequencing reactions, and the other (patient 33) had all three nucleotide sequences identical to HPV16R at the corresponding positions except the one at nt 362, at which an A-to-T change leads to an amino acid change in the E6 (T87S).

DISCUSSION

There are two groups of HPVs based on clinical infection: cutaneous HPVs and mucosal HPVs. These include approximately 200 types of HPVs that have less than 90% similarity with each other at the nucleotide level (2, 26). Cutaneous HPV infection, commonly via abrasion of skin, often causes benign skin warts but, in some rare situations, has been associated with skin cancer (30) and is usually related to HPV5 and HPV8 infection (24). Anogenital HPV infection is the most common type of mucosal HPV infection acquired through sexual intercourse. The oncogenic potential of high-risk HPVs, such as HPV16, -18, and -31, has been well documented in the development of anogenital cancer (27), particularly cancer of the cervix (27, 33) and anus (11). Recent studies suggest that HPV infection may play a role in the development of oral cancer (19, 32, 41), head and neck cancer (12, 31), esophageal cancer (34, 38), lung cancer (8, 16), and colorectal cancer (3, 7). In addition, other reports document the presence of HPV DNA in prostatic tissue (46), sperm cells (20, 21), and breast cancer

TABLE 2. Sequence variations in HPV16 E6 and E2 DNA obtained from PBMCs[a]

Source of HPV16 DNA	Nucleotide variation in HPV16 genome at nt:[b]			
	350	362	442	3684
PBMCs of pediatric HIV patient:				
14	G (V)	A (T)	A (E)	C (T)
23	N	A (T)	A (E)	C (T)
58	G (V)	A (T)	A (E)	C (T)
19	N	A (T)	A (E)	A (K)
16	G (V)	T (S)	A (E)	A (K)
46	N	T (S)	A (E)	A (K)
57	T (L)	T (S)	A (E)	A (K)
33	T (L)	T (S)	A (E)	C (T)
PBMCs of heathy blood donor:				
12	T (L)	T (S)	A (E)	A (K)
17	T (L)	T (S)	A (E)	A (K)
15	G (V)	A (T)	A (E)	C (T)
CaSki cells[b]	G (V)	A (T)	A (E)	A (K)
SiHa cells[b]	G (V)	A (T)	C (D)	A (K)
HPV16R	T (L)	A (T)	A (E)	C (T)

[a] Positions available in the E6 (nt 350, 362, and 442) and E2 (nt 3684) PCR products. N, ambiguous G or T. Letter in parentheses, a resultant amino acid residue in HPV16 E6 or E2 protein.
[b] Both CaSki and SiHa cells are HPV16-positive cervical cancer cell lines.

tissue (43, 45). The latter observations raise questions as to how HPV could localize to these organ tissues given the lack of direct infection and the historical presumption that HPV viremia and hematogenous dissemination do not occur.

Perhaps there is no better interpretation than the finding of HPV DNA in PBMCs to address how HPV could spread and infect epithelial cells in other organs. Previously, several laboratories have demonstrated that HPV DNA could exist in PBMCs of patients with genital HPV infection (29), in the peripheral blood of patients with cervical cancer (15, 17, 28, 40), and in the sera or plasma of patients with cervical cancer (9, 22) or head and neck squamous cell carcinoma (5). However, HPV DNA detected in the peripheral blood has historically been presumed to have originated from metastasized cancer cells in the blood or from virus-containing cell debris being shed into the blood from local HPV infection. Our study demonstrates that the HPV16 genome exists in PBMCs of pediatric HIV patients who acquired HIV infection via transfusion and vertical transmission (one patient with a history of transfusion before sampling) and who were, according to clinical history, sexually naive. Further study demonstrated that the HPV16 genome is also present in PBMCs of "healthy" blood donors, suggesting a potential for transmission via the bloodstream. To our knowledge, these HPV DNA-positive donors had no clinical complaints or history of genital HPV infection when their blood samples were drawn. However, the possible existence of asymptomatic HPV infection in these donors at the time of blood sample donation cannot be excluded. More importantly, the presence of HPV DNA in PBMCs in this study, albeit at a low DNA copy number, is very unlikely to be a result of cross-contamination since extremely stringent criteria had been used to establish HPV DNA positivity (see Materials and Methods) and subsequent HPV sequencing confirmed the detection of unique HPV variants distinct from laboratory strains.

Although the HPV genome replicates as an episome in benign and most preinvasive lesions, it is integrated into the cellular DNA in most cancers. Prior to integration, the episomal, circular viral genome undergoes linearization by a break, which most frequently occurs in the E2 region (18, 39). Thus, the viral E2 gene is often disrupted during HPV DNA integration. In this report, we show that the HPV16 genome in all PBMCs positive for HPV DNA has an intact E2 and thus exists as an episomal form. This conclusion was further supported by the presence of circular HPV16 genome forms with a head-to-tail linkage in all HPV16-positive PBMC DNAs. Most interestingly, the episomal HPV16 genome in the PBMCs described in this report can be grouped into two subgroups based on nucleotide sequence variations in the E6 and E2 regions. Although mutations in nt 350 and nt 442 in the E6 region and nt 3684 in the E2 region of HPV16 have been documented (23, 37, 42), one subgroup of HPV16 genome characterized from PBMCs in our study contains an additional novel mutation at nt 362 (A to T) which has not been reported before. HPV16 intratypic heterogeneity has been an important focus of phylogenetic studies, and the distribution of HPV16 variants has been geographically grouped into five distinct phylogenetic branches: European, Asian, Asian-American, African-1, and African-2 (6, 13). Recently studies suggest that HPV intratypic sequence variation might be a risk factor for the development of high-grade cervical intraepithelial neoplasia (44) and different forms of cervical cancer (4). Specifically, women with HLA-B*44, HLA-B*51, or HLA-B*57 who were infected with the HPV16 E6 variant L83V had an approximately four- to fivefold-increased risk for cervical cancer (47). Thus, it will be interesting to know whether the two subgroups of HPV16 variants identified from PBMCs in our study are biologically different from other common variants in pathogenicity and immunogenicity.

The results from this study have important implications regarding HPV transmission and pathogenesis. However, we were unable to detect HPV transcripts from HPV DNA-positive PBMCs or to define which cell subpopulation (monocytes or lymphocytes) preferentially harbors HPV genomes, indicating that PBMCs likely function as nonpermissive carriers. Although detection of HPV DNA in PBMCs is not synonymous

with the presence of virions in these cells, its association with PBMCs in this study cannot be attributed to malignant lesions as has been previously hypothesized (9, 22, 28, 40). Since PBMCs migrate to sites of tissue inflammation and also take up microorganisms from tissues or the bloodstream, we speculate that PBMCs execute this function for HPV infection, as they do for many other viral infections. Consequently, PBMCs might serve as a source of HPV in the infection of epithelial cells and contribute to their nonsexual spread. However, additional work is needed to confirm this as a possible mode of HPV transmission. Further studies of specimens from linked donor-recipient repositories will be essential to establish a direct linkage.

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一般的名称	①乾燥人フィブリノゲン ②フィブリノゲン加第XIII因子	研究報告の公表状況	Journal of Medical Virology 78(5)693-701,2006	公表国 アメリカ
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研究報告の概要

Vesivirus 属に属する病原性 calicivirus 類は、海洋生態系を循環していると共に、陸生哺乳動物に伝播し且つ陸生哺乳動物間で拡がっている。海生の Vesivirus 疾患がヒトに起こった例としてこれまでの報告されているのは、1 例は海生の Vesivirus の研究をしていた実験科学者で、この科学者は播種性水泡疹を発症し、第 2 の例は海生哺乳類の研究をしていた野外生物学者でこの生物学者は顔面に水疱性発疹ができた。Vesivirus 感染が人の間で拡がっているか否かを評価するために、米国北西部の地域血液検査機関で献血血液の検査を受けた成人及び原因不明の肝炎患者について、Vesivirus の抗体検査及び Vesivirus のウイルス血症の調査を行った。検体は 1996～1999 年の 4 年間に連続して収集されたドナー血漿で以下の様に分類し、ドットブロット分析と 2 つの逆転写 PCR 試験の 3 つの方法で、Vesivirus 遺伝子の試験を行った結果を示す。

①連続の血液スクリーニングで問診及び検査（HBs 抗原、HBc 抗原、HIV-1/2、HIV P24 抗原、HTLV-II、HCV 抗原並びに ALT が 120 IU 未満）に合格した血清（検体数 374、陽性率 12%）。
②問診及び検査で ALT のみが 120 IU 以上で他の項目は全て合格のドナーから採取した連続のサンプル（検体数 350、陽性率 21%）。
③原因不明の肝炎（A-G 以外の肝炎）に罹っているヒトからのサンプルで輸血又は透析に関係するもの（検体数 15、陽性率 47%）。
④原因不明の肝炎（A-G 以外の肝炎）に罹っているヒトからのサンプルで輸血又は透析に関係しないもの（検体数 26、陽性率 19%）。
⑤HBV 又は HCV が原因の肝炎に罹っているヒトからのもの（検体数 10、陽性率 10%）。

さらに、試験を実施した 112 血清検体の内 11 検体（9.8%）が、逆転写 PCR 陽性であり、この内、高 ALT ドナー及び正常値ドナーの陽性率はそれぞれ 11%（検体数 91）及び 4.8%（検体数 21）であった。

これらのデータは、試験を行った血液ドナーの中に、以前に Vesirirus に感染したことを示す抗体陽性の者がおり、またその中には Vesivirus のウイルス血症を有する者もいることを示している。肝炎発症や輸血経験等のない正常ドナーからウイルス血症が発見されたことは、Vesirirus 属への暴露が多様なルートで起きることを示している。これら結果は、人の Vesirirus 感染と疾患との関係をさらに調査することが必要であることを示している。

使用上の注意記載状況・その他参考事項等

代表としてフィブリノゲンHT-Wfの記載を示す。

2. 重要な基本的注意

(1)本剤の原材料となる献血者の血液については、HBs 抗原、抗 HCV 抗体、抗 HIV-1 抗体、抗 HIV-2 抗体、抗 HTLV-I 抗体陰性で、かつ ALT(GPT)値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV 及び HCV について核酸増幅検査（NAT）を実施し、適合した血漿を本剤の製造に使用しているが、当該 NAT の検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した血漿を原料として、Cohn の低温エタノール分画で得た画分から人フィブリノゲンを濃縮・精製した製剤であり、ウイルス不活化を目的として、製造工程においてリン酸トリ-n-ブチル(TNBP)／ポリソルベート 80 処理、濾過膜処理(ナノフィルトレーション)、凍結乾燥の後、60℃、72 時間の加熱処理を施しているが、投与に際しては、次の点に十分注意すること。

報告企業の意見	今後の対応
Vesivirus属感染がヒトの間で広まっていることを示唆する報告である。 Vesivirus属の肝炎に係る病原性は現時点では明確ではないが、米国の血液ドナーの中に、以前にVesivirusに感染したことを示す抗体陽性の者がおり、またその中にはVesivirusのウイルス血症を有する者もいることを示しているため、今後注意深く追加情報をフォローする必要があると考える。	Vesivirus 属に関する追加情報の入手に努める。

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Vesivirus Viremia and Seroprevalence in Humans

Alvin W. Smith,[1]* Patrick L. Iversen,[2] Douglas E. Skilling,[1] David A. Stein,[2] Karin Bok,[3] and David O. Matson[3]

[1]*Department of Biomedical Sciences, College of Veterinary Medicine, Laboratory for Calicivirus Studies, Oregon State University, Corvallis, Oregon*
[2]*AVI Biopharma, Inc., Corvallis, Oregon*
[3]*Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, Virginia*

Pathogenic caliciviruses of the genus *Vesivirus* circulate in oceanic ecosystems and spread to and among terrestrial mammals. Isolation of *Vesivirus* from natural and laboratory infections in humans led to this investigation of *Vesivirus* seroprevalence and viremia. Sera from four groups were tested for antibodies to *Vesivirus* as follows: blood donors whose units were cleared for donation, blood donors whose units were not accepted for donation solely because of elevated blood liver alanine aminotransferase (ALT) concentrations, patients with clinical hepatitis of unknown but suspected infectious cause, and patients with clinical hepatitis of unknown cause but associated with blood transfusion or dialysis. Additionally, sera were tested for *Vesivirus* genome by three methods: dot-blot and two reverse transcription-polymerase chain reaction (RT-PCR) methods. The calculated seroprevalence against *Vesivirus* virions within these groups (N = 765) was 12%, 21%, 29%, and 47%, respectively ($P < 0.001$ for group differences). Additionally, 11 (9.8%) of 112 sera tested yielded RT-PCR amplicons that by nucleotide sequence were distinct from each other and related to known *Vesivirus*. These data indicate that some blood donors in the population tested have serologic evidence of previous *Vesivirus* infection and some also have *Vesivirus* viremia. These results justify further investigation of an association between *Vesivirus* infection and illness in humans. ***J. Med. Virol. 78:693–701, 2006.***

KEY WORDS: calicivirus; *Vesivirus*; human; seroprevalence; viremia

INTRODUCTION

Recent spread of newly recognized human pathogens from animal reservoirs, for example, Nipah virus, coronavirus associated with severe acute respiratory syndrome (SARS), and avian influenza strains, has focused renewed study of viral ecology [Enserink, 2000, 2003; Smith, 2000; Trampuz et al., 2004]. A relatively under-explored potential reservoir of newly emergent human pathogens is the marine environment [Smith et al., 1998b; Culley et al., 2003]. We previously described laboratory and natural marine *Vesivirus* calicivirus infections in humans [Smith et al., 1998a].

Vesivirus is one of four genera in the *Caliciviridae*, the others being *Norovirus* and *Sapovirus*, common causes of gastroenteritis in humans, and *Lagovirus*, widespread in Eurasia in rabbits and hares [Green et al., 2000]. Within the *Vesivirus* genus, serotypes of marine origin form a polyphyletic grouping distinct from those designated feline caliciviruses (FCVs) [Berke et al., 1997]. *Vesivirus* cycles naturally in oceanic reservoirs and a single *Vesivirus* serotype has infected species as diverse as fish, seals, shellfish, swine, cattle, and primates, including humans, and caused disease, sometimes severe, in marine and domesticated food-producing animals and humans [Smith et al., 1998a,b]. Classical virologic investigations established that epidemics of vesicular exanthema of swine virus (VESV) in North America from 1932 through 1959 were caused by serotypes of marine origin [Smith et al., 1978, 1980b; Smith, 2000]. The VESV epidemic serotypes (N = 13) are closely related to serotypes (N > 25) of San Miguel sea lion viruses (SMSVs) also of ocean origin and the few other *Vesivirus* serotypes (N = 5) isolated from terrestrial animals since 1970 [Smith et al., 1978, 1980b; Smith and Boyt, 1990; Neill et al., 1995, 1998; Matson et al., 1996; Reid et al., 1999; Smith, 2000]. Viruses in the marine environment other than caliciviruses also are

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*Correspondence to: Dr. Alvin W. Smith, 105 Magruder Hall, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331. E-mail: calicit@qwest.net

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being discovered, a recent example being the characterization of four possibly new virus families related to caliciviruses at a high taxonomic level [Culley et al., 2003; Lang et al., 2004]. Of interest, the VESV epidemics were controlled, in part, by cooking of raw garbage containing fish and pork scraps fed to swine [Smith et al., 1978, 1980b; Smith and Boyt, 1990].

The FCVs also are in the genus *Vesivirus* and neutralizing antibodies to them occur in marine mammals [Berke et al., 1997; Smith, 2000]. Other *Vesivirus* strains (N = 5) as yet only isolated from terrestrial sources have antigenic and/or genetic properties similar to the *Vesivirus* serotypes often referred as the marine caliciviruses (e.g., [Seal et al., 1995; Barlough et al., 1998; Smith, 2006].

Vesivirus infection can have a multiplicity of clinical outcomes, dependent upon the host and strain, which include encephalitis, hepatitis, myocarditis, pneumonia, spontaneous abortion, and dermatitis (reviewed in [Smith et al., 1998b, 2002a,b; Smith, 2000]). The human cases of marine *Vesivirus* disease which were described previously occurred in association with the North Pacific Basin, one in a laboratory scientist working with a marine *Vesivirus* who experienced a disseminated vesicular exanthem and the second in a field biologist working with marine mammals who had a vesicular exanthem on the face [Smith et al., 1998a]. One strain recovered from these human cases was by genome sequence related most closely to SMSV-5, the serotype known to infect more than 20 animal species and cause different clinical manifestations in them [Smith et al., 1977, 1980a, 1998b; Barlough et al., 1986]. The other strain from a human case was of a new *Vesivirus* serotype. To assess whether *Vesivirus* infections are more widespread among humans, the seroprevalence against *Vesivirus* and possible *Vesivirus* viremia were examined in adults whose donated blood was tested at a northwest-U.S. regional blood laboratory and, because of initial results, in patients with clinical hepatitis of unknown etiology.

MATERIALS AND METHODS

Serum Samples

Sequential, de-identified sera from blood donors were collected over a 4-year period (1996–1999), based upon availability, from the Regional Red Cross Blood Testing Laboratory in Portland, Oregon, which serves eight Western States and was processing samples from about 3,000 units of blood per day. One sample set included blood cleared for donation after clinical and laboratory screening, which included negative test results for hepatitis B surface and core antigens, HIV-1 and 2, HIV P24 antigen, human T-cell lymphotropic virus type 2, hepatitis C antigen, and serum alanine aminotransferase (ALT) levels below 120 International Units (IU). A second, concurrently acquired set of sequential blood specimens was collected from donors whose pre-donation laboratory values differed from the first set only in that the ALT level was ≥120 IU. Based upon testing of these two sets of samples, three additional sets of sera from cases of clinical hepatitis were acquired from an industry source. Two of these sets represented hepatitis cases of unknown but suspected infectious cause. Both were negative for known hepatitis viruses, but differed from each other in that some cases were associated with transfusion or dialysis. Other samples came from hepatitis cases caused by hepatitis B or C virus. The study was approved by the Institutional Review Boards of the authors' institutions.

Antigen Preparation

Antigen for serum antibody detection was generated from four genus *Vesivirus* family *Caliciviridae* serotypes, three SMSVs and one a feline *Vesivirus* prototype, as follows: SMSV-5, originally recovered from blisters on the flippers of a Northern Fur Seal in the Bering Sea and subsequently from skin lesions on a researcher [Smith et al., 1998a]; SMSV-13, recovered from large skin erosions on the flippers and mouth of a California Sea Lion (*Zalophus californianus)* at a marine mammal rescue facility and subsequently shown to cause similar disease in swine and cattle [Berry et al., 1990]; SMSV-17, initially isolated from infected shellfish and an aborted California Sea Lion fetus on the Santa Barbara Channel Islands off the coast of California and subsequently demonstrated to establish persistent infections in experimentally exposed shellfish [Smith, 2000; Burkhardt et al., 2002]; and prototype FCV F9 vaccine strain [Meanger et al., 1992].

Each virus strain was grown in roller bottles to a titer of ~10^7 plaque-forming units per ml in Vero cells. When cytopathology was scored 4+ (~24 h post-inoculation), cells were lysed by freeze-thawing, cellular debris was removed by centrifugation and the supernatant applied to a continuous CsC1 gradient with a mean density of 1.35 g/ml. Virus bands were visible at a density of about 1.36 g/ml and were removed, cleared of CsC1 by dialysis and checked for purity and concentration by direct electron microscopic examination. Optimal dilutions of individual purified virus stocks were determined, then equal parts of each of each SMSV stock were pooled and both preparations (SMSV pool and F9) were retitrated for optimal dilution.

Enzyme Immunoassay (EIA) for Antibody Detection

The EIA utilizing the SMSV pool antigen was described previously [Smith et al., 1998a]. SMSV pool antigen was applied directly to the plate and then incubated with a 1:100 dilution of human serum. Bound immunoglobulin was detected by an alkaline phosphatase-conjugated, anti-human immunoglobulin second antibody (1:40,000 dilution; Sigma, St. Louis, MO). Optical densities (ODs) of the color substrate Blue Phos (KPL, Gaithersburg, MD) were read at 650 nm on a Titertek Multiskan Plus EIA Plate Reader (Titertek, Huntsville, AL). For comparison, sera were tested against wells with no viral antigen. EIA data were

analyzed as P-N values, in which the OD of the antigen-negative well (N) was subtracted from the OD of the well containing viral antigen (P). The OD values of antigen-negative (N) wells were ≤0.005 and most were 0.000. The cut-point for positivity was a P-N ≥0.043. Previous testing [Smith et al., 1998a] demonstrated antibody in EIA-positive samples by Western blot and by preabsorption with SMSV-5 antigen, and that paired sera from cases of Norwalk virus gastroenteritis showed no seroresponse to the SMSV pool. *Vesivirus* typing antisera show broad cross-reactivity of the SMSV-5 capsid antigen with that of other *Vesivirus* serotypes [Kurth et al., 2006].

The FCV antigen also was utilized in the same EIA, in which all other reagents and steps were the same. The cut-point for the FCV antigen also was a P-N value ≥0.043, which was derived by utilizing antigen of assay potency comparable to that of the SMSV pool and a graphical method for estimating cut-point [Matson, 1999].

Vesivirus Nucleic Acid Detection and Characterization

To test the possibility that sera may contain *Vesivirus* genomes, as suggested by the clinical evidence of viremia in cases of *Vesivirus* illness, including humans [Smith et al., 1998a], three complementary methods targeting three *Vesivirus* genomic regions were utilized to detect *Vesivirus* RNA in serum (Fig. 1): *dot blot* for the ORF1 3C protease region, *reverse transcription-polymerase chain reaction (RT-PCR)* for the 3′ terminal region of ORF1 encoding a portion of the viral RNA polymerase or for a portion of the viral capsid protein, and *nucleotide sequencing* of RT-PCR amplicons.

For the *dot-blot method*, a 280-bp biotin-labeled riboprobe (5R.3) specific for the *Vesivirus* 3C protease genomic region (designed from SMSV-5 and corresponding to nts 3,727–4,007 of primate *Vesivirus* prototype *Pan-1*, GenBank number AF091736) was synthesized by in vitro transcription from recombinant plasmid pCN5R.3 (not shown) and probed against 50 ng of total RNA extracted from blood donor serum using Trizol (Invitrogen, Inc., Carlsbad, CA) blotted onto a nylon membrane. Comparison controls also blotted included 20 ng of RNA extracted from CsCl-banded SMSV-5 and 50 ng of total RNA from mouse embryo (Clontech, Inc., Palo Alto, CA). The membrane was probed at 50°C in Northern Max Hyb Buffer (Ambion, Inc., Austin, TX) and washed at high stringency (2X SSC, 1% SDS, 60°C).

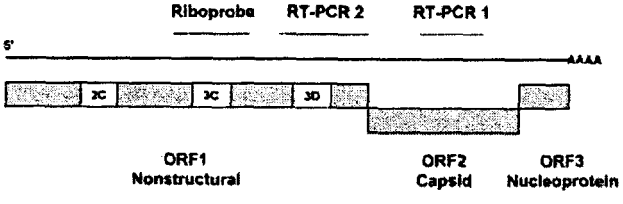

Fig. 1. Genome organization of *Vesivirus* and genome regions detected in this study. The *Vesivirus* genome detection and characterization were directed at two regions of ORF1 and one region of ORF 2 with complementary methods (drawing not to scale).

For one *RT-PCR* (RT-PCR 1), total RNA was extracted from 100 µl of serum using Trizol. A positive control (tissue culture of primate calicivirus) and a negative control (water) were included in each round of extraction in order to control for the quality of the extraction. Extracted RNA was diluted in water containing 0.4 U/µl of RNAsin (Promega, Madison, WI) and used directly in RT-PCR assays. The RNA was amplified with primers D3A 10F (5′-CCAAA GCCAA CAACC GTTGG TTCCA TG-3′, designed from prototype A48 strain, GenBank number U76884, and corresponding to nts 6,962–6,988 of *Pan-1*) and D3A 481R (5′-GTGTA GCAAT CCTGA CAACT TTGCT GG-3′, designed from prototype A48 strain, and corresponding to nts 7,415–7,441 of *Pan-1*) and yielding a product of approximately 480 nt. This RT-PCR utilized Enhanced Avian Reverse Transcriptase (Sigma) according to manufacturer's instructions and Platinum Pfx DNA Polymerase (Invitrogen) also following the manufacturer's recommended protocol. For the second RT-PCR (RT-PCR 2), total RNA from serum was amplified in a similar manner using primers 289rc, 5′-TATGG TGATG ACGGG GTCTA CA-3′ (designed from nucleotides 5,123–5,144 of *Pan-1*), and primer "pre-capsid," 5′-CACCT CACCA CTGAG CCC-3′ (designed from nucleotides 5,650–5,633 of *Pan-1*), and yielding a product of approximately 510 base pairs. RT-PCR amplicons were resolved in a 1% agarose gel and visualized by ethidium bromide under UV light.

For *nucleotide sequencing* of RT-PCR amplicons, RT-PCR products were sequenced directly or after cloning. Cloning used the pGEM-T vector system I (Promega) following the manufacturer's protocol. Recombinants obtained from cloning were screened by PCR using forward and reverse primers targeting the SP6 and T7 polymerase promoters bracketing the multiple cloning cassette. Plasmid DNA from the positive clones was purified by QIA prep Spin Miniprep Kit (Qiagen, Valencia, CA) and sequenced with Sequi Therm EXCEL II Long-read DNA Sequencing Kit (Epicentre Technologies, Madison, WI) on an automated sequencer (ALF Express DNA Sequencer, Amersham Pharmacia, Piscataway, NJ).

Sequence Comparisons

Raw DNA sequences were edited in OMIGA 2.0 (Accelrys, San Diego, CA). Sequences were compared and aligned in CLUSTAL W with prototype sequences retrieved from the NCBI public databases using BLAST searches. Comparison *Vesivirus* sequences included human *Hom-1* (GenBank number U623227), primate *Pan-1* (AF091736), SMSV-2, SMSV-5, SMSV-6, SMSV-7, SMSV-13, and SMSV-14 (U18730, U18731, U18732, U18733, U18734, and U18735), VESV strains A48, C52, E54, I55, and 1934b (U76874, U18738, U18739, U18740, and U18736), bovine (U18741), feline strain F9 (M86379), rabbit (AJ866991), skunk strains 4-1L, 4-2S, and 7-2 (U14668, U14670, and U14672), and

walrus (AF321298). Two sequences of SMSV-1 (U15301 and U14676), called here SMSV-1A and SMSV-1B, and of SMSV-4 (U15302 and U14674), called here SMSV-4A and SMSV-4B, were included.

GenBank numbers of sequences utilized or determined in this study include: for the 3C region riboprobe, DQ300285; for the capsid region amplicon of RT-PCR 1: sample 4, DQ300286; for the polymerase region amplicons of RT-PCR 2: sample N102, DQ300287; sample N104, DQ300288; sample 214, DQ300289; sample 298, DQ300290; sample 310, DQ300291; and sample N330, DQ300292.

Definitions

Healthy blood donors (Normal) are adults volunteering to donate blood and whose donation cleared screening tests and was accepted for donation. Most are repeat donors. Persons with elevated ALT (high ALT) are other blood donors, like the healthy donors, except their blood donation was rejected solely because of the detection of elevated serum ALT values in the blood at the time of donation. Samples from persons with infectious hepatitis (B and C) and hepatitis of unknown etiology (non-A-G hepatitis) came from an industry repository established to discover new causes of human hepatitis.

Statistical Analysis

Optical density (P-N) values were compared between groups by ANOVA and, for correlation, by regression. Difference in estimated prevalence of anti-*Vesivirus* antibodies among groups were assessed by the χ^2 test. The trend of estimated prevalence among groups with increasingly greater evidence of hepatitis was assessed by the Mantel χ^2 for the trend. *P*-values <0.05 were considered significant.

RESULTS

Prevalence of *Vesivirus* Antibody in Different Groups

A total of 374 sera from Normal and 350 sera from high ALT blood donors were tested against both *Vesivirus* antigens. Results from this testing led to obtaining a set of sera from patients with hepatitis (see Materials and Methods) and retesting against the SMSV pool antigen because the estimated prevalence of antibody to both antigens in the high ALT donors was about twice that observed among the Normal blood donors ($P < 0.05$, Yates' corrected χ^2 for each comparison) and mean P-N values were significantly higher among the high ALT donors than among the Normal donors when tested against the SMSV pool antigen but not when tested against the F9 antigen ($P < 0.05$, Student's *t*-test). The sera from all groups were recoded and tested together; the results of this combined testing are presented in Table I.

In the retesting, high ALT donors had a higher prevalence of antibody to both test antigens (21% for the SMSV pool and 14% for F9) than the Normal donors ($P < 0.001$ and $P = 0.026$, respectively). The retesting also yielded a higher mean P-N value among positive samples in the high ALT donors for antibody to the SMSV pool ($P < 0.001$), but not for antibody to F9. Sera from 51 cases of clinical hepatitis had a yet higher estimated prevalence (25%) of anti-SMSV pool antibody

TABLE I. Anti-*Vesivirus* Serum Antibody Among Different Study Groups

	SMSV antigen		Feline CV antigen	
Group[a]	No. positive/no. tested[b]	Mean (SD) P-N of Pos. samples[b]	No. positive/No. tested#	Mean (SD) P-N of Pos. samples
Normal	44/374 (12)[c,d,e,f,l]	145 (243)[h,i,j]	31/374 (8.2)[k]	214 (320)
High ALT	73/350 (21)[c,f,l]	241 (303)[h]	48/350 (14)[k]	192 (250)
Hepatitis cases	13/51 (25)[d]	267 (408)[i]	ND	ND
Non-A-G hepatitis	12/41 (29)[e]	278 (425)[j]	ND	ND
Associated with transfusion or dialysis	7/15 (47)[f,g,l]	102 (65)	ND	ND
Exposures source unknown	5/26 (19)	524 (560)	ND	ND
HBV or HCV hepatitis	1/10 (10)	139 (—)	ND	ND

[a]"Normal" = blood donors cleared for donation by all screening procedures. "Elevated ALT" = blood donors whose units were rejected only because of elevated liver ALT; "Non-A-G hepatitis" = cases of clinical hepatitis suspected to be of infectious etiology, but not linked to any known etiologic agent of hepatitis.
[b]Statistically significant comparisons between groups are indicated by lower-case letters, as follows.
[c]$P = 0.001$.
[d]$P = 0.013$.
[e]$P = 0.004$.
[f]$P < 0.001$.
[g]$P = 0.041$.
[h]$P < 0.001$.
[i]$P = 0.022$.
[j]$P = 0.034$.
[k]$P = 0.026$.
[l]$P < 0.001$.
Tests of proportions between groups by Yates' corrected χ^2 or Mantel χ^2 for the trend. Tests of means of samples reacting positively by Student's *t*-test when variances were normally distributed or by the Kruskal–Wallis H test when significant differences between variances occurred [Dean et al., 1994].