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Asymptomatic Reactivation and Shed of Infectious Varicella Zoster Virus in Astronauts

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Abstract

Varicella zoster virus (VZV) causes varicella (chickenpox), after which virus becomes latent in ganglia along the entire neuraxis. Virus reactivation produces zoster (shingles). Infectious VZV is found in vesicles of patients with zoster and varicella, but virus shed in the absence of disease has not been documented. VZV DNA was previously detected in saliva of astronauts during and after spaceflight, a uniquely stressful environment in which cell mediated immunity (CMI) is temporally dampened. The decline in CMI to VZV associated with zoster led to the hypothesis that infectious VZV would also be present in the saliva of astronauts subjected to stress of space-flight. Herein, not only was the detection of salivary VZV DNA associated with spaceflight validated, but also infectious virus was detected in saliva from 2 of 3 astronauts. This is the first demonstration of shed of infectious VZV in the absence of disease.

Keywords

varicella zoster virus (VZV); human; histochemistry; subclinical; reactivation

INTRODUCTION

Aerosol borne varicella zoster virus (VZV) enters the nasopharynx and replicates in tonsillar T-cells, resulting in viremia and varicella (chickenpox) [Ku et al., 2005]. Virus then becomes latent in cranial nerve, dorsal root and autonomic nervous system ganglia along the entire neuraxis [Mahalingam et al., 1992; Gilden et al., 2001]. Decades later, as cell-mediated immunity to VZV declines, latent VZV can reactivate to produce zoster (shingles) [Miller, 1980], and infectious VZV is present in vesicles. Besides the presence of VZV in vesicles of patients with zoster [Nahass et al., 1995], VZV DNA and infectious virus were detected in the saliva of zoster patients [Mehta et al., 2008].

In addition to the detection of VZV DNA in patients with zoster, VZV DNA was also found in saliva of healthy astronauts during and shortly after spaceflight, indicative of stress-induced

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subclinical virus reactivation [Mehta et al., 2004]. This led to the hypothesis that infectious VZV is present in saliva of otherwise healthy individuals experiencing acute stress. Thus, saliva from healthy astronauts before, during and after spaceflight, was analyzed. Not only VZV DNA, but also infectious virus was detected, proving that VZV, like herpes simplex virus types (HSV-1) 1 [Kaufman et al., 2005] and 2 [Koelle et al., 1992; Raguin and Malkin, 1997] can reactivate and shed infectious virus in the absence of clinical disease.

MATERIALS AND METHODS

Subjects

The Committee for the Protection of Human Subjects of the Johnson Space Center, Houston, TX, approved all human study protocols, and informed consent was obtained from all subjects. Saliva samples were obtained from 3 astronauts before, during and after a 13-day mission.

Sample Collection

A total of 120 saliva samples was obtained from three subjects: 42 saliva samples before launch, 12 saliva samples during spaceflight, and 42 saliva samples after landing. Saliva (1–2 ml) was collected with Salivette kits (Sarstedt, NC) as described [Pierson et al., 2005]. Briefly, cotton rolls were rolled in the mouth until saturated with saliva and returned to the transport vial. Preflight samples were centrifuged at 1303*g* for 10 min and stored at –70°C. In-flight samples were mixed with 1.0 ml biocidal storage buffer (1% SDS, 10 mM Tris–HCl, and 1 mM EDTA) and kept at ambient temperature. After landing, the saliva samples were centrifuged and saliva was stored at –70°C. Post-flight samples were centrifuged at 1303*g* for 10 min. On days 2–6 post-flight, one-half of the saliva sample (~1 ml) was removed for virus isolation, while the remaining sample was stored at –70°C. On days 7–15 post-flight, all of the saliva sample was stored at –70°C.

A total of 12 blood samples (3–5 ml) was collected into EDTA containing vacutainer (Becton Dickinson, Franklin Lakes, NJ) by venous puncture. Cells were removed by centrifugation (1303*g* for 10 min) and plasma was stored at –70°C.

Antibody Testing

The antibody titers to HSV–1 and VZV were determined by indirect immunofluorescence. Coverslips containing acetone fixed HSV1 and VZV-infected human diploid fibroblast cells were prepared commercially (Bion Enterprises, Park Ridge, IL), and incubated with twofold dilutions of plasma in phosphate buffered saline (PBS). After PBS washes, bound antibody was detected with FITC-conjugated anti-human IgG as directed by the supplier (Bion Enterprises). The endpoint titer was defined as the highest dilution of plasma that revealed positive immunofluorescence. All plasma samples were coded and analyzed simultaneously.

Extraction of DNA From Saliva and PCR

Saliva samples were concentrated to 0.2 ml by centrifugation through a Microsep 100 K filtration unit (Filtron Technology Corp., Northborough, MA). Polyacryl microcarrier gel (20 μl; Molecular Research Center, Inc., Cincinnati, OH) was added and DNA was extracted by affinity chromatography on silica-matrix (Qiagen, Inc., Chatsworth, CA). DNA was dissolved in 50 μl nuclease-free water (Amresco, Solon, OH). Quantitative real-time PCR was performed in a TaqMan 7700 sequence detector (Perkin Elmer Biosystems, Boston, MA) using fluorescence-based simultaneous amplification and product detection. Primers and probes for VZV, HSV-1 and glyceraldehyde 6-phosphate dehydrogenase (GAPdH) are shown in Table I. PCR assays were performed in 50-μl volumes containing 2× TaqMan Universal PCR Master Mix (Perkin–Elmer, Norwalk, CT) and 2 μl of extracted DNA as described [Cohrs et al., 2000]. Standard curves were generated with diluted VZV DNA (0–10^6 copies) extracted from virus-infected cells [Gilden et al., 1982]. Each sample was analyzed in triplicate.

Virus Isolation and Culture

Saliva (~1 ml) samples obtained 2–6 days after landing were diluted to 2 ml with complete-Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA) and 1× antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA). One-day old human fetal lung fibroblast (HLF) cell cultures were spin-inoculated as described [Weinberg et al., 1996] with the following modifications. The inoculated cultures were centrifuged at 1,000*g* for 15 min at room temperature, incubated at 37°C for 60 min, and diluted with 10 ml complete-DMEM. After overnight incubation and at 3-day intervals, the medium was replenished.

Immunohistochemistry

Replicate cell cultures of HLF were inoculated with saliva from the three subjects obtained 2–6 days after landing. When CPE developed (3 days post infection), the cells were fixed for 20 min at 4°C in fresh 4% paraformaldeyhde in PBS, permeabilized for 10 min in methanol–acetone (50:50), blocked for 60 min in 3% bovine serum albumin in TE (150 mM NaCl, 20 mM Tris–HCl), and incubated for 60 min with 1:2,000 dilution of rabbit anti-VZV-IE63 [Mahalingam et al., 1996] or a 1:1,000 dilution of rabbit anti-HSV–1-ICP22 [Blaho et al., 1997]. Rabbit antibody was bound to secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG; 1:10,000 dilution; Invitrogen) and detected colometrically with NBT/BCIP (Roche, Nutley, NJ).

Cell Culture DNA Extraction

HLF cells (1–5 × 10^6) inoculated with saliva were mechanically dislodged and collected by centrifugation (1,000*g*, 10 min, 4°C). Cell pellets were resuspended in 0.2 ml TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA), and total DNA was extracted by affinity chromatography on a silica matrix (DNeasy, Qiagen).

VZV Genotype Analysis

PCR-based diagnostic assays [Loparev et al., 2007] were performed on DNA extracted from HLF cultures that developed a CPE after inoculation with saliva. Single nucleotide polymorphorism (SNP) in VZV open reading frame (ORF) 38 (ORF38), ORF54 [LaRussa et al., 1992] and ORF62 (positions 106,262 and 107,252) were determined using FRET (fluorescent resonance energy transfer)-based PCR performed on LightCycler (Roche, Pleasanton, CA) as described [Loparev et al., 2000]. The PCR forward and reverse primers (p22R1f and p22R1r) were designed to amplify a 447-bp fragment (positions 37,837 to 38,264) of VZV ORF 22, and sequence variation within this amplicon was conducted as described [Loparev et al., 2007]; additional sequence analysis at ORF1, ORF21, and ORF50 was performed to refine the identification of the genotype. All VZV genomic locus numbers are based on the published nucleotide sequence for the Dumas strain of VZV [Davison and Scott, 1986].

RESULTS

VZV DNA in Astronaut Saliva

VZV DNA was detected in saliva from 2 of 3 astronauts (Table II). In subject 1, 6 of 12 samples obtained during spaceflight contained 120 to 2,500 copies of VZV DNA per ml saliva; after landing, 1,250 copies of VZV DNA were present on day 2, 45 copies on day 3 and 110 copies on day 5. All samples taken 6–15 days after touchdown were negative for VZV DNA. In subject

2, 5 of 12 samples obtained during spaceflight contained 18 to 650 copies of VZV DNA per ml saliva; after landing, 560 copies of VZV DNA were present in saliva on day 2, 340 copies on day 4, 45 copies on day 5, and 23 copes on day 6. All samples taken 7–15 days after touchdown were negative for VZV DNA. None of 42 preflight saliva samples taken from the three subjects contained VZV DNA. All saliva samples contained amplifiable GAPdH DNA sequences.

VZV and HSV-1 Antibodies in Astronaut Plasma

The VZV IgG antibody titer in all three subjects (Table II) was 1:80 at the annual medical exam (AME) 2–3 months before spaceflight. Ten days before liftoff, the anti-VZV IgG antibody titer increased to 1:320 in subject 1 and to 1:640 in subject 2. This increase over AME baseline was also detected at landing. Two weeks later, the anti-VZV IgG antibody titer decreased to 1:80 in both subjects 1 and 2. Subject 3 did not develop any increase in anti-VZV antibody titer in response to spaceflight. HSV-1 antibodies were also detected in all three subjects, but at a much reduced level (Table II). The anti-HSV-1 antibody titer for subject 1 and at AME was 1:10 and remained at 1:10 during the entire study period. The anti-HSV-1 antibody titer for subject 2 at AME was 1:10, rose to 1:40 10 days before launch and was 1:40 at landing, and again 2 weeks later. No anti-HSV-1 antibody was found in subject 3 during AME, was 1:10 10 days before launch, and at landing and 2 weeks later.

Saliva VZV DNA and Plasma VZV Antibody in Healthy Control Subjects

Saliva and plasma were analyzed from 14 healthy subjects (9 men and 5 women, 34–70 years old) three times over a 2-week interval for VZV DNA and circulating anti-VZV IgG antibodies (Table III). No VZV DNA was detected in any of the 42 saliva samples. Plasma from all control subjects contained anti-VZV antibodies. The anti-VZV IgG antibody titer in 7 of 15 subjects (50%) demonstrated a twofold variation from day 1 during the 2-week study period. No healthy subject demonstrated more than a twofold increase or decrease in circulating anti-VZV antibody titer over the 2-week study period.

Infectious VZV in Saliva of Astronauts

A virus induced CPE was seen in the HLF cell cultures inoculated with saliva from astronauts 1 and 2 on the second day after landing. None of the other 8 HLF cell cultures inoculated with saliva from subjects 1 and 2 obtained 3–6 days after landing developed a CPE, and none of the 5 HLF cell cultures inoculated with saliva from subject 3 obtained 2–6 days after landing developed a CPE. HLF cell cultures with a CPE (subjects 1 and 2 at 2 days after landing) showed positive immunostaining for VZV (Fig. 1). No other HLF cell cultures were VZV positive, and no cultures were positive after staining with rabbit anti-HSV-ICP22 antibody (data not shown).

PCR analysis of DNA extracted from HLF cell cultures that developed a CPE after inoculation with the astronaut saliva from subjects 1 and 2 obtained on day 2 after landing and which were positive for VZV by immunostaining confirmed the presence of VZV (Table IV). PCR analysis on DNA extracted from all HLF cell cultures was negative for HSV-1.

Genotypic Analysis of VZV Isolates

Both VZV isolates obtained from the astronauts' saliva lacked a *Pst*I restriction endonuclease site within ORF38, but contained *Bgl*I and *Msp*I sites within ORFs 54 and 62, respectively, indicating wild-type VZV. The DNA sequenced between positions 37,837 to 38,264 matched the Dumas strain of VZV, sufficient to classify the new isolates within the European genotype of VZV (Table V).

DISCUSSION

Current findings that infectious VZV can be isolated from saliva of otherwise healthy astronauts shortly after flight in space extends the previous report of VZV DNA in astronaut saliva where real-time PCR detected VZV DNA from 24 to 25,000 copies per ml of saliva in 4 of 8 (50%) astronauts on two short-duration (10–13 days) missions [Mehta et al., 2004]. This current study found 18–2,500 copies of VZV DNA per ml of saliva in 2 of 3 subjects after a 13-day mission. Of the 15 post-flight saliva samples tested for virus recovery, only the samples obtained on day 2 post-flight from subjects 1 and 2 yielded infectious VZV. These samples contained 560 to 1,250 VZV DNA copies per ml of saliva. Since infectious VZV was recovered from saliva that contained 560 copies of VZV DNA per ml, the two in-flight saliva samples which contained 650 and 2,500 copies of VZV DNA per ml (flight day 9) might also have been expected to contain infectious VZV. Genotypic comparison of VZV isolated from a zoster vesicle with VZV cultured from astronaut saliva revealed a unique thymine to cytosine transition at nucleotide position 107,252 (data not shown) thereby excluding laboratory contamination. Future genotypic analysis of virus from cultured and matched uncultured salivary VZV DNA will be useful to identify possible common virus sources.

In subject 3, neither infectious virus nor VZV DNA was found in saliva, and there was no rise in anti-VZV IgG antibody titer. Since subject 3 had developed zoster 10 years earlier, he was probably protected from virus reactivation during flight in space by a boost in cell-medicated immunity to VZV that is known to occur after a single episode of zoster [Hayward et al., 1991].

Spaceflight provides a unique situation where healthy individuals are continuously exposed to confined, unfamiliar environments consisting of extreme isolation from family in a crowded living/work space, lack of privacy and sleep deprivation all within a microgravity setting that demands accurate and precise physical and mental functions. Consistent with an environment of acute stress, increased levels of ACTH and cortisol [Stein and Schluter, 1994; Stowe et al., 2001], and a decreased CMI response to various antigens have been associated with spaceflight [Taylor and Janney, 1992]. Acute stress along with a transient depression in immunological surveillance also increases herpesvirus reactivation [Freeman et al., 2007]. Two other human herpesviruses (cytomegalovirus and Epstein–Barr virus) were shown to reactivate in astronauts associated with spaceflight [Payne et al., 1999; Mehta et al., 2000; Pierson et al., 2005]. The detection of infectious virus in saliva of otherwise healthy individuals in the current study conclusively demonstrates asymptomatic shed of VZV. While saliva was not analyzed for HSV-1 by PCR, the slight rise in circulating anti-HSV-1 IgG antibody titer concomitant with the fourfold rise in anti-VZV IgG antibodies suggests that HSV-1 reactivation may have also occurred, although the amount of virus produced was not sufficient to be detected in tissue culture.

The finding of infectious VZV in saliva without reported oral lesions is noteworthy. VZV most likely reaches the nasopharynx via transaxonal transport along special visceral efferent fibers after reactivation from the geniculate (seventh cranial nerve) ganglion, a common site of VZV latency and reactivation [Sweeney and Gilden, 2001; reviewed in Mueller et al., 2008]. Once present in the oral region, reactivated VZV could replicate in lymphoid tissue and reach the tonsillar surface through penetrating epithelial cells [Ku et al., 2005].

To our knowledge, this is the first report documenting asymptomatic reactivation with shedding of infectious VZV in saliva. Thus, like HSV-1 and HSV-2, VZV can reactivate in the absence of clinical disease [Corey, 1993; Kaufman et al., 2005]. A major limitation encountered with studies involving spaceflight is the small sample size, and future studies on Earth are required to determine the extent of asymptomatic VZV reactivation in the normal population.

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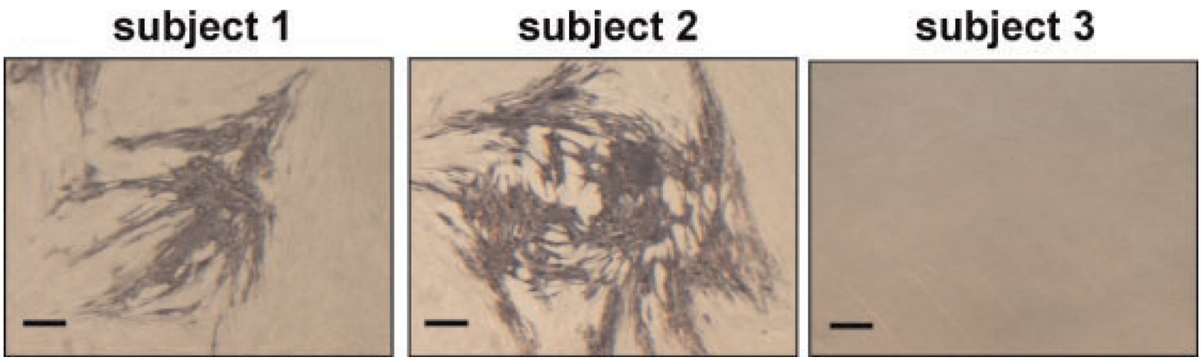

Fig. 1.

Recovery of infectious VZV from astronaut saliva. Human lung fibroblast cells cultures were inoculated with saliva from astronauts obtained on day 2 after landing. Typical herpesvirus plaques were seen in cultures inoculated with saliva from subjects 1 and 2, but not with saliva from subject 3. The plaques stained with anti-VZV antibody, but not with anti-HSV-1 antibody (not shown). magnification bar = 0.2 mm.

TABLE I

PCR Oligonucleotide Primers and Probes

Target	Type	Sequence (5′-3′)
VZV	F	CGC GTT TTG TAC TCC GGG
	R	ACG GTT GAT GTC CTC AAC GAG
	Probe[a]	TGG GAG ATC CAC CCG GCC AG
HSV-1	F	TGG TAT TGC CCA ACA CTT TCC
	R	GCG CCA GGC ACA CAC AT
	Probe	CGT GTC GCG TGT GGT
GAPdH	F	CAA GGT CAT CCA TGA CAA CTT TG
	R	GGC CAT CCA CAG TCT TCT GG
	Probe	ACC ACA GTC CAT GCC ATC ACT GCC A

F, forward primer; R, reverse primer.

[a]Probes contain 5′-FAM and 3′-BHQ labeled (IDT, Coralville, IA).

TABLE II

Saliva VZV DNA and Serum Antibody Titer in Astronauts

		VZV DNA copies/ml saliva subjects			VZV antibody titer subjects			HSV1 antibody titer subjects		
Sample	**Flight days**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
AME		n.s.[a]	n.s.	n.s.	1:80	1:80	1:80	1:10	1:10	n.d.[b]
Pre-flight	–133	0	0	0						
Pre-flight	–131	0	0	0						
Pre-flight	–129	0	0	0						
Pre-flight	–127	0	0	0						
Pre-flight	–125	0	0	0						
Pre-flight	–123	0	0	0						
Pre-flight	–121	0	0	0						
Pre-flight	–119	0	0	0						
Pre-flight	–125	0	0	0						
Pre-flight	–117	0	0	0						
Pre-flight	–115	0	0	0						
Pre-flight	–113	0	0	0						
Pre-flight	–111	0	0	0						
Pre-flight	–109	0	0	0						
Pre-flight	–10	n.s.	n.s.	n.s.	1:320	1:640	1:80	1:10	1:40	1:10
In-flight	1	n.s.	n.s.	n.s.						
In-flight	2	224	18	0						
In-flight	3	0	247	0						
In-flight	4	0	0	0						
In-flight	5	128	0	0						
In-flight	6	0	0	0						
In-flight	7	200	0	0						
In-flight	8	0	0	0						
In-flight	9	2,500	650	0						
In-flight	10	0	75	0						
In-flight	11	450	0	0						
In-flight	12	0	0	0						

		VZV DNA copies/ml saliva subjects			VZV antibody titer subjects			HSV1 antibody titer subjects		
Sample	Flight days	1	2	3	1	2	3	1	2	3
In-flight	13	120	23	0						
Post-flight	14	n.s.	n.s.	n.s.	1:320	1:320	1:80	1:10	1:40	1:10
Post-flight	15	1,250	560	0						
Post-flight	16	45	0	0						
Post-flight	17	0	340	0						
Post-flight	18	110	45	0						
Post-flight	19	0	23	0						
Post-flight	20	0	0	0						
Post-flight	21	0	0	0						
Post-flight	22	0	0	0						
Post-flight	23	0	0	0						
Post-flight	24	0	0	0						
Post-flight	25	0	0	0						
Post-flight	26	0	0	0						
Post-flight	27	0	0	0	1:80	1:80	1:80	1:10	1:40	1:10
Post-flight	28	0	0	0						

AME, annual medical exam.

[a] No sample available.

[b] Antibody not detected.

TABLE III

Saliva VZV DNA and Plasma Antibody Titer in Healthy Subjects

		VZV DNA copies/ml saliva			VZV antibody titer		
Sex	**Age**	**Day 1**	**Day 7**	**Day 14**	**Day 1**	**Day 7**	**Day 14**
M	34	0	0	0	1:40	1:80	1:80
M	40	0	0	0	1:40	1:80	1:40
M	40	0	0	0	1:80	1:80	1:80
F	43	0	0	0	1:80	1:80	1:80
F	48	0	0	0	1:40	1:80	1:80
F	49	0	0	0	1:160	1:80	1:160
F	50	0	0	0	1:80	1:80	1:80
M	54	0	0	0	1:160	1:160	1:160
F	54	0	0	0	1:80	1:160	1:80
M	55	0	0	0	1:80	1:160	1:80
M	62	0	0	0	1:80	1:80	1:80
M	64	0	0	0	1:80	1:80	1:80
M	65	0	0	0	1:160	1:160	1:80
M	70	0	0	0	1:80	1:80	1:80

TABLE IV

PCR Analysis of HFL Cells Inoculated With Astronaut Saliva

Days post flight	Subject 1		Subject 2		Subject 3	
	VZV	HSV-1	VZV	HSV-1	VZV	HSV-1
2	+	–	+	–	–	–
3	–	–	–	–	–	–
4	–	–	–	–	–	–
5	–	–	–	–	–	–
6	–	–	–	–	–	–

TABLE V

Genotype Analysis of VZV Isolates

	Open Reading Frame														
	1				21		22				38	50	54	62	
Sample	685	789	790	791	33,725	33,728	37,902	38,055	38,081	38,177	69,349	87,841	95,241	106,262	107,252
Subject 1	G	T	T	T	C	C	A	T	A	G	A	T	T	T	T
Subject 2	G	T	T	T	C	C	A	T	A	G	A	T	T	T	T
VZV (Dumas)	G	T	T	T	C	C	A	T	A	G	A	T	T	T	T
VZV (HJO)	G	T	T	T	C	C	A	T	A	G	A	T	T	T	T
VZV (Parental Oka)	A	C	C	C	C	C	G	C	C	A	G	T	C	T	T

Genotypic Marker
European
Japanese
Vaccine