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Pediatric Epstein-Barr Virus Carriers With or Without Tonsillar Enlargement May Substantially Contribute to Spreading of the Virus

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Background. Human-to-human transmission of the persistent infection establishing Epstein-Barr virus (EBV) occurs via saliva. Tonsils act as an important portal of entry and exit of EBV. The contagiousness of pediatric EBV carriers and the role played by tonsillar enlargement (TE) are not known.

Methods. We compared EBV shedding in mouthwash samples from pediatric EBV carriers with or without TE to that in mouthwash samples from pediatric patients with infectious mononucleosis (IM), the symptomatic form of primary infection if delayed after the age of 5 years. EBV DNA was quantified by polymerase chain reaction, and contagiousness was assessed using the cord lymphocyte transformation assay.

Results. EBV carriers with TE shed EBV DNA at an almost similar frequency (although in lower amounts) as pediatric patients with acute IM but more frequently (P < .001) and in higher amounts (P = .038) than EBV carriers without TE. EBV DNA levels in mouthwash samples from EBV carriers with TE mirrored levels in tonsils and gradually declined after tonsillectomy. Almost half of the mouthwash samples from pediatric EBV carriers contained infectious EBV.

Conclusions. Pediatric EBV carriers—in particular, those with TE—may considerably contribute to the spreading of EBV in industrialized countries.

More than 90% of the adult population carry Epstein-Barr virus (EBV), a human gammaherpesvirus that establishes latency in memory B cells to persist in the host [1]. Thus, EBV is one of the most successfully spreading viruses [2]. The members of the population involved in the spreading of EBV are only incompletely defined.

Human-to-human transmission of EBV occurs mainly via saliva [1]. Palatine tonsils act as an important site used by EBV for invasion of the host and as a reservoir [3, 4]. Oropharyngeal shedding of EBV via saliva is linked to virus reactivation from latency [1] and is increased in immunocompromised hosts, including human immunodeficiency virus type 1–infected patients [5] and allograft recipients [6]. Although these individuals are spreaders of EBV, the bulk of overall EBV spreading likely originates from the much larger immunocompetent population. Reactivation of EBV in immunocompetent hosts may be initiated by terminal differentiation of EBV-carrying memory B cells to plasma cells in tonsils [7]. Recurrent or chronic infection within tonsils results in their enlargement, and the effect that tonsillar enlargement (TE) in EBV carriers has on EBV shedding is largely unknown.

Adults with acute infectious mononucleosis (IM) invariably shed large amounts of EBV DNA and/or infectious particles in saliva for several months [8–10], in contrast with healthy adults, who shed EBV at varying percentages and in smaller amounts [10–14]. Although acute IM manifests in up to 50% of adolescents and 70% of adults experiencing primary EBV infection
[15, 16], the vast majority of primary EBV infections occur during early childhood [17, 18]. Then, EBV acquisition seems to exhibit an asymptomatic or an unspecific clinical course. Little is known about EBV shedding by pediatric patients with IM or EBV carriers: 2 studies from Africa showed that EBV DNA was present in saliva from up to 90% of children [19, 20], and 1 study from Japan detected EBV DNA in saliva from 38% of healthy children <6 years of age with unknown EBV carrier status [21].

In the present study, we aimed to determine the frequency and amount of oral EBV shedding among pediatric EBV carriers with or without TE in comparison to that in children with acute and convalescent IM from an industrialized country. Our results indicate that children constitute a potentially important source of EBV transmission.

METHODS

**Subjects and study design.** Children with acute IM or EBV seropositivity undergoing tonsillectomy for TE or children with no TE (NTE) undergoing other elective surgery at University Children’s Hospital of Zurich were prospectively enrolled after written informed consent was obtained. Mouthwash and peripheral blood samples were collected at enrollment, and mouthwash samples from patients with IM or from EBV carriers with TE were obtained 3 and 6 months later. From carriers with TE we obtained the tonsils. Diagnosis of acute IM was established on the basis of the presence of fever, TE, hepatosplenomegaly, and generalized lymph node enlargement, plus detectable immunoglobulin M (IgM) to viral capsid antigen (VCA) and absent immunoglobulin G (IgG) to EBV nuclear antigen in serum (Immuno-DOT; GenBio). EBV carriers were defined as EBV-seropositive children (presence of IgG to VCA in the absence of IgM to VCA). Fifty-seven (60%) of the recruited patients with TE and 14 (50%) of those with NTE were EBV seropositive and were included in the study. Indications for tonsillectomy were obstructive sleep apnea syndrome (84%) and recurrent tonsillitis (16%). These children had no history of IM within the past 12 months. The study protocol was approved by the institutional ethics committee.

**Peripheral blood.** Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from venous blood by means of Vacutainer CPT tubes (BD), in accordance with the manufacturer’s instructions (Applied Biosystems).

**Palatine tonsils.** Tonsillar mononuclear cells (TMCs) were collected by Ficoll-Isopaque density gradient centrifugation (Leucosep; Greiner Bio-One) after dissociation of the tissue.

**Mouthwash samples.** Samples from patients with TE or NTE were obtained during anesthesia before surgery by rinsing the mouth with 5 mL of phosphate-buffered saline or, at follow-up visits and from patients with IM, after gargling with 5 mL of phosphate-buffered saline. To detect infectious virus in the cell-free compartment [8, 12, 22], debris and bacteria were removed by low-speed centrifugation (1260 g for 10 min). The supernatant was stored at −80°C until use, whereby a fraction was supplemented with 10% heat-inactivated fetal calf serum before being stored for later use in transformation assays. After thawing, the samples were passed through a filter with a pore size of 0.45 μm (Millipore).

**DNA extraction.** DNA was extracted from PBMCs or tonsils (20–100 mg/sample) and, after addition of herring sperm DNA (Invitrogen), from plasma or mouthwash samples by means of the QIAamp DNA Mini kit (Qiagen) [23]. DNase treatment was performed in accordance with the manufacturer’s instructions (Applied Biosystems).

**Quantitative polymerase chain reaction assay.** EBV DNA levels were determined by the TaqMan (Applied Biosystems) real-time polymerase chain reaction (PCR) technique, using 2 PCR primer-probe systems targeting the conserved EBV BamHI W region [23] and the conserved LMP1 exon 3 region [24]. Serial dilutions of a plasmid containing the targeted sequence were included in every PCR run as an internal control and for calibration [23]. The BamHI W system has a higher sensitivity than the LMP1 system and, after correction for multiple copies of the BamHI W repeats, correlated with the values determined by LMP1 quantitative PCR (qPCR) [24]. Values <2 DNA copies/reaction (the limit of detection) were regarded as negative [3, 23, 24]. The number of cells was quantified by qPCR targeting the single-copy human hydroxymethylbilane synthase gene (HMBS) [3, 4].

**Cell culture and virus stock.** Primary cells and the EBV-producer cell line B95.8 [25] were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum, 1% l-glutamine, and 1% penicillin-streptomycin (Gibco) [26].

**Transformation assay.** Cord blood mononuclear cells (CBMCs) were isolated by Ficoll separation. CBMCs (2 × 10⁶) and mouthwash samples were mixed 1:1 in a total volume of 1 mL and spinoculated for 1 h at 800 g at 24°C [8, 20]. After washing with RPMI 1640, CBMCs were incubated in 96-well plates at 37°C. Half of the medium was changed every 3 days for 6 weeks, when growth of cells indicated CBMC transformation. For each CBMC donor, transformation was controlled by inoculation with B95.8 supernatant [8, 25].

**Statistical analysis.** The χ², Kruskal-Wallis, and Mann-Whitney U tests (Prism 4; GraphPad Software) were used to compare groups regarding the frequency of detection and levels of EBV DNA. The Spearman correlation coefficient was used to assess correlations between EBV DNA levels in distinct compartments. Differences with P < .05 were regarded as statistically significant.
RESULTS

Frequency of detectable EBV DNA in mouthwash, PBMC, and plasma samples from pediatric patients with IM and EBV carriers. Patients with acute IM exhibit TE and shed EBV via saliva [8, 9, 11, 27, 28]. We asked whether pediatric EBV carriers with TE shed EBV in saliva more frequently than do carriers with NTE and whether this is mirrored in peripheral blood. Therefore, by qPCR we assessed and compared the frequencies of detectable EBV DNA in mouthwash, PBMC, and plasma samples from 9 children with acute IM (age range, 2.9–13.9 years; median age, 6 years; mean age, 7.3 years), from 57 EBV carriers with TE (age range, 2.8–14.8 years; median age, 6.0 years; mean age, 6.3 years), and from 14 EBV carriers with NTE (age range, 1.5–15.8 years; median age, 8.8 years; mean age, 8.3 years).

We detected EBV DNA in all mouthwash (n = 9), PBMC (n = 9), and plasma (n = 7) samples from patients with acute IM (Figure 1). Partly in contrast, we detected EBV DNA in 47 mouthwash samples (88%) and 30 PBMC samples (57%) from 53 carriers with TE but in no plasma samples (0%) from 57 carriers with TE. In greater contrast, we detected EBV DNA in 7 mouthwash samples (50%) from 14, in 6 PBMC samples (46%) from 13, and in no plasma samples (0%) from 14 carriers with NTE (Figure 1). Thus, EBV carriers with TE had detectable EBV DNA in mouthwash samples at a frequency similar to that in patients with acute IM and at a frequency significantly higher than that in carriers with NTE (P = .001). This suggests that TE is associated with more frequent EBV shedding than NTE.

EBV DNA levels in mouthwash samples from pediatric patients with IM and EBV carriers. We wondered whether EBV DNA levels in mouthwash samples from carriers with TE differed from those in mouthwash samples from patients with acute IM or from carriers with NTE. We found EBV DNA levels between 55 and 21,592 copies/mL of mouthwash among patients with acute IM, between <2 and 14,744 copies/mL of mouthwash among carriers with TE (P = .002), and between <2 and 1377 copies/mL of mouthwash among carriers with NTE (P = .038, for the comparison with carriers with TE) (Figure 2). The median EBV DNA level in mouthwash samples from carriers with TE was ~17-fold lower than that for patients with acute IM but was ~5-fold higher than that for carriers with NTE (Figure 2).

Next, we asked whether these differences were reflected in PBMCs. We found EBV DNA levels between 1.8 and 1249 copies/10^4 PBMCs for patients with acute IM, between 0.1 and 71 copies/10^4 PBMCs for carriers with TE (P < .001), and between 0.4 and 56 copies/10^4 PBMCs for carriers with NTE (P = .001) (Figure 2). The median EBV DNA level in PBMCs from carriers with TE was ~150-fold lower than that for patients with acute IM but was rather similar to that in PBMCs from carriers with NTE (Figure 2).

These observations suggest that TE in carriers is associated with increased EBV DNA shedding, a process apparently not mirrored by increased EBV DNA levels in PBMCs.

EBV DNA levels in mouthwash samples, tonsils, and PBMCs from pediatric EBV carriers with TE. We investigated whether mouthwash EBV DNA levels mirror the levels in autologous enlarged tonsils. We found a highly statistically significant correlation between mouthwash levels and levels in TMCs (r = 0.65; P < .001) (Figure 3A), suggesting that the amount of EBV in tonsils may dictate the amount shed in saliva. Furthermore, we found a highly statistically significant correlation between EBV DNA levels in tonsils and PBMCs (r = 0.71; P < .001) (Figure 3B). This suggests that the amount of EBV in enlarged tonsils may influence the amount circulating in PBMCs.

Next, we asked whether EBV DNA levels in mouthwash samples and PBMCs correspond in carriers with TE. We found a highly statistically significant correlation (r = 0.69; P < .001) (Figure 3C). Thus, the amount of EBV in enlarged tonsils seems to influence the amount in saliva and PBMCs. We found no correlation between EBV DNA levels in mouthwash and PBMC samples from patients with acute IM (r = −0.04; P = .69) (data not shown) but did find a correlation for carriers with NTE (r = 0.57; P = .044) (data not shown), suggesting that the amounts of EBV shed in saliva and in PBMCs are closely related in EBV carriers, in striking contrast to patients with acute IM.

EBV DNA levels in mouthwash samples from pediatric patients with IM and EBV carriers over time. We asked whether the frequency of detectable EBV DNA and EBV DNA levels would diminish after tonsillectomy, analogous to what is observed in adult convalescent IM patients [29]. The proportion
Figure 2. Epstein-Barr virus (EBV) DNA levels in mouthwash samples (left panel) or peripheral blood mononuclear cells (PBMCs) (right panel) from pediatric patients with acute infectious mononucleosis (IM) (n = 9), pediatric EBV carriers with tonsillar enlargement (TE) (n = 57), and pediatric EBV carriers with no TE (NTE) (n = 14). EBV DNA levels were determined by quantitative polymerase chain reaction. Horizontal lines in the box plot indicate medians; the bottoms and tops of the boxes indicate lower and upper quartiles, respectively; and the brackets indicate minimal and maximal values.

of pediatric patients with IM who had detectable EBV DNA in mouthwash samples diminished over time, from 100% during the acute phase, to 6 (87%) of 7 patients 3 months later, and to 3 (60%) of 5 patients 6 months later (Figure 4A). EBV DNA levels ranged from 55 to 21,592 copies/mL of mouthwash during the acute phase and then decreased to a range of <2 to 931 copies/mL of mouthwash 3 months later (P = .005) and to a range of <2 to 268 copies/mL of mouthwash 6 months later (P = .004) (Figure 4A). Among carriers with TE, the frequency of detectable EBV DNA in mouthwash samples diminished from 88% at tonsillectomy to 19 (66%) of 29 children 3 months later and to 8 (47%) of 17 children 6 months later. EBV DNA levels ranged from <2 to 14,744 copies/mL of mouthwash at tonsillectomy and then decreased to a range of <2 to 3400 copies/mL of mouthwash 3 months later (P = .035) and to a range of <2 to 2905 copies/mL of mouthwash 6 months later (P < .001) (Figure 4B). These results illustrate a decrease in the frequency of detectable EBV DNA and in EBV DNA levels shed in saliva by children after IM or tonsillectomy due to TE.

Transformation of CBMCs by mouthwash. To investigate the infectivity of saliva, we determined transformation efficiency using CBMCs. First, we ascertained that mouthwash does not markedly influence the transformation efficiency of inoculated EBV (data not shown).

Second, we compared EBV DNA levels with the presence of infectious EBV using serial dilutions of B95.8 culture supernatant. Supernatant containing at least 4500 EBV DNA copies/mL resulted in a transformation efficiency of 100%. The inoculated amount of EBV DNA and the transformation efficiency correlated significantly (r = −0.82; P = .034) (Figure 5A).

Third, we investigated the transformation capacity of mouthwash samples from 6 patients with IM. The mean EBV DNA level in mouthwash samples from patients with acute IM resulting in transformation of CBMCs was 3330 EBV DNA copies/mL (range, 120–50,300 EBV DNA copies/mL). Five samples (83%) resulted in transformation of CBMCs, whereby the inoculated amount of EBV DNA and the transformation efficiency correlated significantly (r = −0.85; P = .033) (Figure 5B). The mean EBV DNA level in mouthwash samples 1–6 months after acute IM was 120 EBV DNA copies/mL (range, 2–736 EBV DNA copies/mL). Four samples (67%) resulted in transformation of CBMCs (Figure 5B). Thus, saliva from the majority of patients with acute IM or during the first 6 months thereafter contains infectious EBV with transformation capacity.

Fourth, we asked whether mouthwash samples from carriers with TE are infectious. The mean EBV DNA level in 10 mouthwash samples corresponded to 400 EBV DNA copies/mL (range, 20–27,510 EBV DNA copies/mL). Four samples (40%) resulted in transformation of CBMCs (Figure 5C). We also tested mouthwash samples from these 10 individuals 3–6 months after tonsillectomy. The mean EBV DNA level was 36 EBV DNA copies/mL (range, 2–4500 EBV DNA copies/mL). Three samples (30%) resulted in transformation of CBMCs (Figure 5C).

Fifth, we performed DNAse digestion of mouthwash samples from 8 patients with IM and 3 carriers with TE to distinguish between total detectable EBV DNA (including naked fragments) and encapsidated DNA (suggesting infectious virus) [30]. Digestion with DNase I reduced total EBV DNA levels determined by qPCR by 90%–95%, which were still detectable...
in 4 of 8 mouthwash samples from patients with IM and in the 3 mouthwash samples from carriers with TE with the highest EBV DNA levels. Three of these 4 mouthwash samples from patients with IM and 2 of the 3 mouthwash samples from carriers with TE with detectable EBV DNA after DNAse digestion induced CBMC transformation. This indicated that mouthwash from patients with IM and from carriers with TE contain infectious EBV.

Taken together, mouthwash samples from a considerable proportion of carriers with TE before and 3–6 months after tonsillectomy as well as from carriers with NTE contain infectious EBV with transformational potential. These results suggest that EBV carriers represent a considerable source of infectious EBV, irrespective of TE.

**DISCUSSION**

This study investigated oral EBV shedding by pediatric EBV carriers with or without TE versus pediatric patients with IM from an industrialized country. We found that (1) pediatric carriers with TE orally shed EBV at a frequency almost similar to that of the invariably shedding pediatric patients with acute IM, although in lower amounts; (2) oral shedding is more frequent and occurs in larger amounts in carriers with TE than with NTE; (3) EBV DNA levels in mouthwash samples from carriers with TE mirror levels in tonsils; (4) oral EBV DNA shedding by carriers with TE decline after tonsillectomy; and
(5) almost half of mouthwash samples from carriers contain infectious EBV. Our results indicate that children constitute an important source of EBV transmission and may considerably contribute to the spreading of EBV in industrialized countries.

A major finding was that the vast majority of carriers with TE shed EBV DNA orally. The frequency of 88% was as high as the up to 90% reported among children from Africa [19] and was almost as high as the 100% among pediatric patients with acute IM found here. Children in Africa were found to shed higher EBV DNA levels than their mothers [19]. Oral EBV DNA shedding by adult carriers from industrialized countries ranged from 50% to 100% [14, 21, 31, 32]. Here, pediatric carriers with TE orally shed EBV significantly more frequently than did carriers with NTE. These results argue that pediatric carriers in industrialized countries may orally shed EBV less frequently than those in developing countries and that TE, similar to malaria [33], results in an increased frequency of shedding. Further indication that TE augments oral EBV DNA shedding is provided by our observation of significantly higher levels in mouthwash samples from carriers with TE than with NTE. Nevertheless, the levels in mouthwash samples from carriers with TE were significantly lower than those in mouthwash samples from acute IM patients. This suggests that, at least in children, maximal oral EBV shedding occurs in primary EBV infection manifesting as IM. In patients with IM, replication of EBV seems to follow distinct patterns relative to those in EBV carriers, independently of TE—as indicated by the detection of EBV DNA in the extracellular and cellular compartments of the peripheral blood of patients with IM, in contrast with the detection (if any) in the cellular compartment only for carriers, as shown here.

Another finding of interest was that EBV DNA levels in mouthwash samples from carriers with TE mirrored the levels in their tonsils and correlated with levels in PBMCs. Our observation of a similar strong correlation between levels in mouthwash samples and PBMCs for carriers with NTE but not for patients with acute IM again indicates that EBV replication patterns differ between primary infection and the carrier state. The results also indicate that in pediatric carriers the amount of EBV shed in saliva and the level of EBV in PBMCs are tightly

Figure 5. Transforming Epstein-Barr virus (EBV) in culture supernatants of wild-type EBV strain B95.8 and in mouthwash samples in relation to EBV DNA levels. A, Wild-type EBV strain B95.8. B, Pediatric patients with acute infectious mononucleosis (IM) and 1–6 months later (IM 1 + 6m). C, Pediatric EBV carriers with tonsillar enlargement (TE) before tonsillectomy (TEat OP) and 3–6 months after tonsillectomy (TE +3 to 6m). D, Pediatric EBV carriers with no TE (NTE). In panel C, only 1 of 4 samples without transformation and no detection of EBV DNA and only 1 of 2 samples without transformation and 36 EBV DNA copies/mL are visible. qPCR, quantitative polymerase chain reaction.
related, in striking contrast to the situation for acute IM. In adult carriers, Yao et al [31] also found a correlation between EBV DNA levels in mouthwash samples and those in PBMCs, but Hadinoto et al [29] found no correlation between EBV in saliva and peripheral blood memory B cells from 8 subjects. Although the number and percentage of B cells circulating in blood, the biological activity of tonsils, the number of upper respiratory tract infections, the time elapsed since primary EBV infection, and possibly EBV shedding [19] are different between children and adults, the EBV DNA levels in PBMCs from our pediatric carriers were similar to those reported for adults [14, 34, 35]. Thus, it is unclear whether the correlation between orally shed EBV DNA and levels in PBMCs are specific for pediatric carriers.

The gradual decay of oral EBV DNA shedding by carriers after tonsillectomy corroborates the potential of TE in contributing to the shedding and spreading of EBV. The decay resembles that seen in pediatric patients with IM during convalescence, when TE diminishes. The decay observed here in pediatric patients with IM is similar to that seen in adult patients with IM in a recent study by Hadinoto et al [29] but contrasts with findings of other studies showing shedding of constant EBV DNA levels for at least 6 months [9, 10].

Almost half of the pediatric EBV carriers with viral DNA in mouthwash samples shed infectious EBV. This is relevant to the circulation of EBV in the community. The infectious potential of mouthwash samples from carriers did not correlate with EBV DNA levels. These findings contrasted with those for supernatants of wild-type EBV cultures and mouthwash samples from patients with acute IM. Fafi-Kremer et al [9] suggested that EBV shed by adults after acute IM is less infectious. Thus, one possible explanation for the disparity between acute IM and convalescent IM or the EBV carrier state in children observed here could relate to infectious properties rather than solely to the quantity of EBV shed.

It is well established that EBV DNA levels determined by qPCR need not correspond to numbers of infectious EBV particles [30, 36]. Unencapsidated EBV DNA may be a confounder that results in overestimation of the amount of EBV, as has been demonstrated using DNAase digestion [30, 36–39]. DNAase digestion and CBMC transformation by mouthwash samples from children with IM or TE provided evidence that DNAase-resistant (ie, contagious) EBV is present in mouthwash from pediatric patients with IM or TE. On the basis of the transformation of CBMCs, infectious EBV was demonstrated in mouthwash samples from children with IM, TE, or NTE. In pediatric EBV carriers, contagiousness was independent of EBV DNA levels, in contrast to patients with IM. Notably, mouthwash samples from single carriers with NTE and without detectable EBV DNA transformed CBMCs. This implies that, despite the high sensitivity of the qPCR assay (lower limit of detection, 2 EBV DNA copies/mL), infectious particles were present in mouthwash.

In conclusion, our findings suggest that pediatric EBV carriers may represent an important source of EBV spreading, especially among young children, given their frequent oral shedding of infectious virus and their rather frequent exchange of saliva resulting from their age-related behavior.

References

EBV Spread by Children


