MAJOR ARTICLE

A Cohort Study among University Students: Identification of Risk Factors for Epstein-Barr Virus Seroconversion and Infectious Mononucleosis

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Background. A vaccine against Epstein-Barr virus (EBV) infection is in clinical trials. Up-to-date information on risk factors for EBV infection and infectious mononucleosis (IM) among young adults is required to inform a vaccination strategy.

Methods. We carried out a prospective study on a cohort of university students. All EBV-seronegative students were asked to report symptoms of IM and were followed up 3 years later to undergo repeat EBV testing and to complete a lifestyle questionnaire. EBV typing was performed for these subjects, as well as for students who were EBV seropositive at enrollment and for additional students with IM.

Results. A total of 510 students (25%) who took part in the study were EBV seronegative when they entered the university; 110 (46%) of these experienced seroconversion while at the university, 27 (25%) of whom developed IM. Penetrative sexual intercourse was a risk factor for EBV seroconversion ($P = .004$), but neither condom use nor oral sex significantly altered the rate of seroconversion. EBV type 1 was significantly overrepresented in IM, compared with silent seroconversion ($P = .001$).

Conclusions. Our findings suggest that acquisition of EBV is enhanced by penetrative sexual intercourse, although transmission could occur through related sexual behaviors, such as "deep kissing." We also found that EBV type 1 infection is significantly more likely to result in IM. Overall, the results suggest that a large EBV type 1 load acquired during sexual intercourse can rapidly colonize the B cell population and induce the exaggerated T cell response that causes IM. Thus, IM could, perhaps, be prevented with a vaccine that reduces the viral load without necessarily inducing sterile immunity.

Infectious mononucleosis (IM) is common among adolescents and young adults in Western society. The disease is caused by primary Epstein-Barr virus (EBV) infection, and persons with infection commonly present with fever, sore throat, lymphadenopathy, and fatigue [1]. The symptoms of IM are believed to be immunopathological in nature and caused by cytokine release from the large numbers of EBV-specific activated T cells present in the blood and tissues during acute-phase IM [2–5]. There is no specific treatment for IM, and although it is generally self-limiting, the severity of illness varies from a mild flulike illness to a prolonged and debilitating disease with symptoms persisting for up to 6 months. Rare chronic and fatal cases occur. In addition, EBV is associated with certain malignancies, and previous IM is a risk factor for the development of Hodgkin disease (reviewed in [6]).

EBV infection usually occurs subclinically in persons during childhood, establishes a persistent infection in B lymphocytes with low-level virus excretion in saliva, and is transmitted through close contact [7]. EBV has also been detected in both male and female genital secretions, suggesting that the virus can be transmitted through sexual contact [8–10].

In nonindustrialized countries, virtually all children are infected with EBV by 2 years of age [11], but many children in Western society are protected from early infection, presumably because of high standards of hy-

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giene. These children are liable to become infected later in life, when IM may develop. Thus, IM is mainly a disease of the affluent in industrialized countries [12].

Early studies on EBV infection in university students and army recruits in the United States and the United Kingdom found that 26%–74% of primary EBV infections in young adults resulted in IM [13–16]. Risk factors for developing IM rather than a subclinical primary EBV infection have not been fully elucidated; however, it has long been suggested that the transmission of a large amount of virus is important in the pathogenesis of IM [17]. In this instance, because the symptoms of IM are T cell–mediated, the initial viral load must determine the level of T cell response and thereby determine whether seroconversion is silent or manifests as IM. In support of this, a recent study showed that viral loads in IM were significantly higher in persons with more severe symptoms, and increased viral load correlated directly with increased levels of activated T cells [18]. In contrast, Silins et al. [19] studied 3 cases of subclinical primary infection and demonstrated viral loads as high as those in cases of IM.

Two distinct types of EBV, 1 and 2 (EBV-1 and EBV-2; also called A and B), have been recognized [20] that show 70%–85% sequence homology [21], and >90% of adults worldwide are persistently infected with either EBV-1 or EBV-2. A limited number of small epidemiological studies investigating the geographical distribution of EBV types show that type 1 is more prevalent worldwide than type 2, although type 2 is more common in Africa than in the United States and Europe [22]. In these studies, dual infection was shown to be mainly restricted to immunocompromised persons [23–25]. In vitro, EBV-1 induces growth transformation of B cells more efficiently than EBV-2 [26], but no type-specific disease associations have been demonstrated (reviewed in [27]).

An EBV vaccine designed to induce neutralizing antibodies against the viral receptor protein, gp350, is being tested in a phase II clinical trial [28]. The initial aim of the vaccine is to prevent IM; however, to develop an evidence-based vaccination program, it is important to have up-to-date information about the risk factors that lead to IM rather than asymptomatic seroconversion. The present study was undertaken to define these risk factors by obtaining follow-up data on seroconversion and lifestyle and/or life events in a cohort of EBV-seronegative students during 3–4 years in college.

MATERIALS AND METHODS

Study participants. Students who enrolled at Edinburgh University in 1999 and 2000 and who were pursuing a course of study lasting for ⩾4 years (honors degree programs in Scotland generally last for 4 years) were asked to join the study. After giving informed consent, the students donated a blood sample for EBV testing.

Students whose test results for EBV were negative were asked to report symptoms suggestive of IM (sore throat, fever, lymphadenopathy, and fatigue) to the study doctor. All initially seronegative students were followed up with at the end of their third year or at the beginning of their fourth year of college and were asked to donate another blood sample for EBV testing and to complete a confidential questionnaire about lifestyle and life events experienced while in college. This included information on living circumstances, medical history (including a specific question about IM), travel, smoking, alcohol consumption, exercise, and sexual relationships.

To increase the number of samples analyzed for EBV type, detection of EBV 1 and 2 was carried out not only on the above students but also on enrollment samples from 1504 EBV-seropositive students and from all consenting students who reported to the University Health Service with IM during the study period (i.e., both students within and not within the cohort). This study was approved by the Lothian Ethics Committee.

EBV serological testing. Serum or plasma samples were initially tested for IgG antibodies to Epstein-Barr viral capsid antigen by routine ELISA (Sigma). Samples with a negative result were checked by indirect immunofluorescence assay [29]. Equivocal serum samples were also tested for IgG antibodies to Epstein-Barr viral nuclear antigens by anticomplimentary immunofluorescence [30]. Seronegativity was defined as a negative result for anti–viral capsid antigen IgG by ELISA and immunofluorescence assay, as well as a negative result for Epstein-Barr viral nuclear antigens (when tested). IM was diagnosed when serum IgM antibodies to viral capsid antigen were detected by indirect immunofluorescence assay (ATI Atlas) with or without a positive monospot test result (Microgen Bioproducts).

EBV-1 and EBV-2 detection. DNA was extracted from PBMCs with the Easy DNA kit (Invitrogen) in accordance with the manufacturer's instructions. The type of EBV (1 or 2) in each PBMC sample was determined by nested PCR amplification across a type-specific region of the Epstein-Barr viral nuclear antigens *3C* gene [31].

The primers used amplified both EBV-1 and EBV-2 sequences in the same reaction, and therefore, both types were detected with equal sensitivity. Briefly, DNA (500 ng–1 μg) was amplified in a primary reaction mix containing 1 × PCR reaction buffer (50 mM of KCl, 10 mM of Tris-HCl [pH, 9.0], and 0.1% Triton X-100) plus 1.5 mM of $MgCl_2$; 200 μM each of dATP, dTTP, dGTP, and dCTP; 1 μM each of primary primer; and 1 U of *Taq* polymerase (Promega). The cycling conditions consisted of 1 cycle of 5 min at 94°C, 40 cycles of 30 s at 94°C, 90 s at 45°C, and 120 s at 72°C, and a final cycle of 5 min at 72°C. The primary product (2.5–5 μL) was further amplified in a second reaction mix containing the secondary primers

Table 1. Risk of Epstein-Barr virus (EBV) seroconversion during college for 241 students, by characteristic.

Characteristic	No. (%) who remained EBV negative during college	No. (%) who experienced EBV seroconversion in college	Prevalence ratio (95% CI)
Sex			
Male	51 (56)	40 (44)	1.0
Female	80 (53)	70 (47)	1.1 (0.8–1.4)
Age at college enrollment, in years			
<19	90 (56)	71 (44)	1.0
⩾19	41 (51)	39 (49)	1.1 (0.8–1.5)
Tonsillectomy			
No	126 (55)	102 (45)	1.0
Yes	5 (38)	8 (62)	1.4 (0.9–2.2)
Stressful events during college[a]			
No	76 (54)	65 (46)	1.0
Yes	55 (55)	45 (45)	1.0 (0.7–1.3)
Travel outside of the United Kingdom during college			
No	11 (58)	8 (42)	1.0
Yes	120 (54)	102 (46)	1.1 (0.6–1.9)
Experienced allergies during college			
No	72 (56)	57 (44)	1.0
Yes	59 (53)	53 (47)	1.1 (0.8–1.4)
All students	131 (54)	110 (46)	…

NOTE. Percentages are calculated separately according to the categories of characteristics, from which prevalence ratios are calculated.

[a] Stressful events include family and relationship, financial, or work-related problems.

under the exact same conditions. Final products were separated on a 2% agarose gel containing ethidium bromide and visualized under UV light.

Statistical analysis. Statistical analyses were conducted to examine the association between a variety of demographic, behavioral, and sexual factors and the risk of EBV seroconversion. The prevalence of EBV seroconversion was calculated for the cohort overall and also separately, according to the categories of the risk factors, from which prevalence ratios (as estimates of relative risks) were calculated. Prevalence ratios, rather than ORs, were calculated to avoid violation of the rare disease assumption required for the latter, because EBV positivity was relatively common among the students [32]. Prevalence ratios were also calculated to identify risk factors for IM versus asymptomatic seroconversion. Wald-based 95% CIs were estimated around each relative risk, and Fisher's exact tests were undertaken to test significance [33]. All P values presented were 2-sided. All analyses were conducted using Stata software, version 8.0 (Stata).

RESULTS

A total of 2006 students donated blood samples on enrollment in college, 510 (25%) of whom were EBV seronegative. All 510 seronegative students were reapproached after 3 years in college, and 241 (47%) donated a second blood sample and completed a lifestyle questionnaire (table 1). Of the 241 students, 38% were male, 62% were female, and the age range was 20–29 years (mean, 20 years 6 months). Students among the 241 who gave a second sample were more likely to be female, compared with students among the 269 who did not ($P < .001$); those in the former group were marginally younger (6 weeks difference in average age) but were not significantly different from those in the latter group regarding sexual activity before college enrollment ($P = .12$).

Of the 241 students who returned for a follow-up visit at 3 years, 110 (46%) had experienced EBV seroconversion during their time in college, whereas 131 (54%) remained EBV seronegative. The percent of students who experienced seroconversion during college was similar by sex and age (table 1). Among those who experienced EBV seroconversion, 27 (25%) reported that they had developed IM (medical confirmation was available for 25 of the 27), whereas the remainder seroconverted without clinical illness (table 2). These data give an average annual seroconversion rate of 15.2% and an IM rate of 3.7% among students who were initially EBV seronegative, and an estimated 3.9% seroconversion rate and 0.95% IM rate for the overall cohort, assuming that the students who returned for follow-up are representative of all seronegative students. The probability of

Table 2. Risk of developing infectious mononucleosis (IM) for 110 students who experienced Epstein-Barr virus seroconversion during college, by characteristic.

Characteristic	No. (%) who experienced seroconversion without IM	No. (%) who experienced seroconversion with IM	Prevalence ratio (95% CI)
Sex			
Male	28 (70)	12 (30)	1.0
Female	55 (79)	15 (21)	0.7 (0.4–1.4)
Age at college enrollment, in years			
<19	53 (75)	18 (25)	1.0
⩾19	30 (77)	9 (23)	0.9 (0.5–1.8)
Tonsillectomy			
No	79 (77)	23 (23)	1.0
Yes	4 (50)	4 (50)	2.2 (1.0–4.8)
Stressful events during college[a]			
No	48 (74)	17 (26)	1.0
Yes	35 (78)	10 (22)	0.8 (0.4–1.7)
Travel outside of the United Kingdom during college			
No	6 (75)	2 (25)	1.0
Yes	77 (75)	25 (25)	1.0 (0.3–3.4)
Experienced allergies during college			
No	43 (75)	14 (25)	1.0
Yes	40 (75)	13 (25)	1.0 (0.5–1.9)
All students	83 (75)	27 (25)	…

NOTE. Percentages are calculated separately according to the categories of characteristics, from which prevalence ratios are calculated.

[a] Stressful events include family and relationship, financial, or work-related problems.

seroconversion during college being associated with IM was similar for males (30%) and females (21%) and for those <19 years of age (25%) and ⩾19 years of age (23%).

A total of 220 students (91%) reported that they engaged in some form of sexual activity during college. The questionnaire distinguished 3 categories with respect to sexual activity: none (21 students), kissing only or genital contact without penetrative sexual intercourse (35 students), and genital contact with penetrative sexual intercourse (185 students). Six students (29%) from the first category, 10 (29%) from the second, and 94 (51%) from the third experienced EBV seroconversion (table 3). There was a statistically significant difference ($P = .004$) in the seroconversion rate between the 185 students who had penetrative sexual intercourse (51%) and the 56 who did not (29%). This suggests that penetrative sexual intercourse may increase the risk of EBV transmission. The questionnaire also asked about condom use and oral sex among the 185 students who had penetrative sexual intercourse; the EBV seroconversion rate was lower, but not significantly so ($P = .27$), among those who reported always using a condom (46%), compared with those who did not (55%) and was also lower, but not significantly so ($P = .62$), among those who had not had oral sex (45%), compared with those who had (52%).

Our analyses of symptomatic versus asymptomatic seroconversions need to be interpreted with caution, because only 27 of 241 students experienced EBV seroconversion with IM in college. The extent to which seroconversion occurred with symptoms of IM rather than silently was not significantly greater among those who had penetrative sexual intercourse (26%), compared with those who had not (19%) (table 4). (If the retrospective data on students who were already seropositive at the time of college entry are also included, the effect of sexual intercourse on the risk of IM, compared with silent seroconversion, is highly significant). The IM seroconversion rate was very similar among those who did and did not always use a condom, but, based on very small numbers, was not significantly greater ($P = .40$) among those who had oral sex (28%), compared with those who did not (13%).

EBV-1 and/or EBV-2 sequences were successfully amplified from 840 student samples, of which 294 were from students with IM, and 546 were from those who experienced silent seroconversion. The overall distribution of EBV-1 and EBV-2 in the study population was as follows: 672 (80%) had EBV-1 infection, 121 (14%) had EBV-2 infection, and 47 (6%) had dual infection with types 1 and 2. This distribution of EBV types differed significantly between students with IM (254 [86%] had EBV-1 infection, 22 [8%] had EBV-2 infection, and 18 [6%] had dual infection) and those with silent seroconver-

Table 3. Risk of Epstein-Barr virus (EBV) seroconversion, by sexual activity.

Sexual activity	No. (%) who remained EBV negative during college	No. (%) who experienced EBV seroconversion during college	Prevalence ratio (95% CI)
Penetrative sexual intercourse	91 (49)	94 (51)	1.0
No penetrative intercourse	40 (71)	16 (29)	0.6 (0.4–0.9)[c]
Genital contact without penetrative intercourse	25 (71)	10 (29)	0.6 (0.3–1.0)[b]
Not sexually active	15 (71)	6 (29)	0.6 (0.3–1.0)
Condom use[a]			
Never or seldom	42 (45)	52 (55)	1.0
Always	49 (54)	42 (46)	0.8 (0.6–1.1)

NOTE. Percentages are calculated separately according to the categories of sexual activity from which prevalence ratios are calculated.

[a] Analyses of condom use restricted to students who had penetrative sex.
[b] $P < .05$.
[c] $P < .01$.

sion (418 [77%], 99 [18%], and 29 [5%]), with EBV-1 infection being overrepresented in IM, compared with silent seroconversion (heterogeneity, $P < .001$; EBV-1 vs. EBV-2 and dual infection, $P < .001$) (table 5).

In addition to the risk factors outlined above, the questionnaire included detailed questions on medical events, physical illness, psychological and/or mental illness, illicit drug and/or alcohol use, stressful events (including family and/or relationship, financial, or work-related problems), medication, and allergies experienced during college. Vaccination with bacille Calmette-Guérin during college showed a positive association with EBV seroconversion ($P = .01$) but not with IM. No other types of vaccination or other parameters examined showed an association with EBV seroconversion or IM (table 1).

DISCUSSION

A gp350-based EBV vaccine aimed at preventing IM is in phase II clinical trials [28]. However, it is not known whether this will induce sterile immunity or whether sterile immunity is required to prevent IM. Furthermore, there is very little recent information on numbers of students finishing school who are susceptible to IM and risk factors for disease development. No logical vaccine strategy can be planned without this information. Our study was designed to gain up-to-date data on EBV seroprevalence and to identify groups at risk for IM. We recruited >2000 university students, 25% of whom were EBV seronegative. Three years later, 47% of the seronegative students returned to donate another blood sample. Although these students differed from those who did not return for follow-up with respect to sex, and marginally, to age, these variables were not among those found to be associated with risk of IM, and there were no significant differences regarding history of sexual intercourse. Thus, as far as we can determine, there does not appear to be a nonresponse bias.

Published data on EBV prevalence and IM, mainly dating from the 1970s, showed that 3%–5% of university students in the United States and Europe develop IM annually [13–15], and this can lead to significant loss of study time. Our study gave an annual IM rate of 0.95%, which, although lower than the incidence reported in university students by earlier studies [13–15], is similar to the figure reported for US Army recruits (0.9%) in the 1970s [16]. The lower IM rate in our students and US Army recruits reflects the lower percentage of EBV-seronegative individuals at enrollment in these studies; all studies, including ours, show a seroconversion rate of 10%–20% per year for persons who were initially seronegative.

The largest of the early studies [15] found that 27 (45%) of 60 students who seroconverted during college did so with symptoms of IM. This result contrasts with our study; we found that 27 (25%) of 110 students who seroconverted developed IM. However, although a figure of ~50% is commonly quoted for the incidence of IM among young adults experiencing primary EBV infection, published studies show a range of 26%–74% [13–16].

As reported previously by us and our colleagues [34], we have found higher rates of seropositivity among sexually active students, as well as evidence (although it is not significant) of a protective effect of condom use. These findings support the direct transmission of EBV though genital secretions. However, EBV can be detected in the saliva of most seropositive individuals (in 59%, according to 1 study [35]), and salivary contact alone is clearly sufficient for the efficient spread of EBV among children. In contrast, EBV can only be found at low levels and only in a minority of genital secretion samples [8–10] (R.T.

Table 4. Risk of developing infectious mononucleosis (IM) for 110 students who experienced Epstein-Barr virus seroconversion during college, by sexual activity.

Degree of sexual activity in college	No. (%) who experienced seroconversion without IM	No. (%) who experienced seroconversion with IM	Prevalence ratio (95% CI)
Penetrative sexual intercourse	70 (74)	24 (26)	1.0
No penetrative intercourse	13 (81)	3 (19)	0.7 (0.3–2.2)
Genital contact without penetrative intercourse	9 (90)	1 (10)	0.4 (0.1–2.6)
Not sexually active	4 (67)	2 (33)	1.3 (0.4–4.3)
Condom use[a]			
Never or seldom used condoms	39 (75)	13 (25)	1.0
Always used condoms	32 (76)	10 (24)	1.0 (0.5–2.0)

NOTE. Percentages are calculated separately according to the categories of sexual activity, from which prevalence ratios are calculated.

[a] Analyses of condom use restricted to students who had penetrative sex.

and D.H.C., unpublished data). Therefore, it seems reasonable to assume that EBV is usually transmitted orally, even in adults, and this view is supported by the common clinical features of IM (sore throat and cervical lymphadenopathy) that strongly suggest an oral route of transmission. If this is the case, then our data could be explained in the instance that an increased dose of EBV is transmitted by deep kissing during penetrative sexual intercourse and that this enhances virus transmission (with or without IM) in young adults. In addition, in our study, those who had penetrative sexual intercourse also had more sexual partners (an average of 3 or 4) than those who did not (an average of 1 or 2), thus increasing the risk of transmission by either the oral or sexual route. Waldeyer's ring is assumed to be the primary site of EBV infection, and this lymphoid tissue reaches its maximum size during early childhood and thereafter regresses (at ~7 years of age for the nasopharyngeal [adenoid] and tubal tonsils, and 14 years for the palatine tonsil) [36]. Thus, it is possible that EBV infection occurs more easily in children, with a small dose of salivary virus effecting silent transmission.

The distribution of EBV-1 and EBV-2 found among the student population in this study is similar to the findings of smaller studies [27]. There has previously been some suggestion that EBV-1 infection is overrepresented in primary infections that lead to IM [27], and our larger study shows this to be significantly so. This finding is consistent with the differences in biological activity previously noted between EBV-1 and EBV-2 in tissue culture experiments in which EBV-1 shows an enhanced ability to stimulate B cell activation and immortalization [26]. In vivo, this property could lead to more rapid colonization of the B cell pool, enhance the T cell response, and thereby increase the likelihood of IM (we plan to examine other type-specific associations elsewhere; C.D.H., A.J.S., K.F.M., H.W., K.M., R.T., S.R., M.C., D.H.C., K. Britton; unpublished data). Several small studies have suggested an overrepresentation of EBV-1 in EBV-associated Hodgkin disease [27], and because IM is a significant risk factor for Hodgkin disease, our results would be supportive of such an association.

Taken together, the results of this study suggest that IM is most likely to occur when a seronegative individual is infected with a large amount of a virus that has an enhanced ability to stimulate B cell proliferation. Thus, both quantitative and qualitative differences in the initial viral inoculum influence whether primary EBV infection is silent or manifests as IM. Based on

Table 5. Distributions of Epstein-Barr virus (EBV) type in infectious mononucleosis (IM) and silent seroconversion.

Type of seroconversion	No. (%) with seroconversion to EBV-1	No. (%) with seroconversion to EBV-2	No (%) with dual infection with EBV-1 and EBV-2
Silent	418 (77)	99 (18)	29 (5)
With IM	254 (86)	22 (8)	18 (6)
Total	672 (80)	121 (14)	47 (6)

NOTE. Heterogeneity, $P < .001$. Percentages are calculated separately according to the type of seroconversion.

these results, we suggest that a vaccine that reduces the level of viral infection and/or replication during primary infection could be sufficient to prevent IM; in this scenario, sterile immunity would not be required.

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