Prospective studies of antibodies to multiple Epstein-Barr virus (EBV) proteins and EBV neutralizing antibodies in the same individuals before, during, and after primary EBV infection have not been reported. We studied antibody responses to EBV in college students who acquired primary EBV infection during prospective surveillance and correlated the kinetics of antibody response with the severity of disease. Neutralizing antibodies and enzyme-linked immunosorbent assay (ELISA) antibodies to gp350, the major target of neutralizing antibody, reached peak levels at medians of 179 and 333 days after the onset of symptoms of infectious mononucleosis, respectively. No clear correlation was found between the severity of the symptoms of infectious mononucleosis and the peak levels of antibody to individual viral proteins or to neutralizing antibody. In summary, we found that titers of neutralizing antibody and antibodies to multiple EBV proteins increase over many months after primary infection with EBV.

Epstein-Barr virus (EBV) infects more than 90% of the human population worldwide and is the primary cause of infectious mononucleosis (IM) (1). In a recent prospective study of university students with primary EBV infection, 77% developed IM, 12% had atypical symptoms that did not meet the criteria for IM, and 11% were asymptomatic (2). Symptoms of IM included sore throat (93%), cervical lymphadenopathy (76%), fatigue (66%), upper respiratory tract distress (61%), headache (47%), anorexia (44%), fever (42%), and myalgias (41%). The median duration of illness was 10 days and ranged from 3 to 66 days.

The diagnosis of IM is usually based on the presence of typical symptoms in combination with a positive heterophile antibody response. Heterophile antibodies are not directed against EBV proteins but are antibodies that have been absorbed to guinea pig kidney and that agglutinate sheep, horse, or cow red blood cells. These antibodies persist for up to 1 year after primary EBV infection. Specific antibody tests that recognize EBV proteins have been developed for the diagnosis of primary infection. Detection of IgM antibody to the EBV viral capsid antigen (VCA) is the most useful test result since these antibodies are detectable only during the first 2 to 3 months after the onset of symptoms. In contrast, detection of IgG antibody to EBV VCA at the onset of symptoms is not useful since this antibody may not develop until several weeks after the onset of symptoms and persists for the lifetime of the individual. Less commonly, demonstration of seroconversion from a negative to a positive EBV VCA IgG or EBV nuclear antigen (EBNA) IgG has been used for diagnosis.

Few studies have measured levels of antibody to specific EBV proteins over time after the initial infection. Most studies have looked at sera obtained at one or a few time points per patient from multiple persons after IM, rather than obtaining samples from the same patients at multiple time points for several months after their illness. Using the former approach, levels of IgG antibody to EBV VCA were found to be present at the onset of IM, to rise for a few weeks thereafter, and to fall gradually and persist for life (3, 4). Using the same approach, Henle et al. found that antibody to EBNA2 appeared before antibody to EBNA1 after primary infection; EBNA2 titers peaked and eventually declined to very low or undetectable levels, while levels of antibody to EBNA1 rose weeks to months after those to EBNA2 and persisted for the life of the patient (5).

Neutralizing antibodies that inhibit EBV-induced proliferation or transformation of B cells have been detected within the first 3 weeks of IM (3, 6). Neutralization antibody titers rose after initial diagnosis of IM, reached a plateau by 3 to 4 months, and persisted at the plateau level for at least 2 years (3). Neutralizing antibody titers were present in most adults many years after primary EBV infection (3, 6).

We are unaware of studies in which antibody to multiple EBV proteins and neutralizing antibody have been quantified over time in the same individuals before, during, and after primary EBV infection. Therefore, we analyzed sera obtained prospectively from university students who had been followed for a median of 3 years for development of primary EBV infection (2). We sought to determine how long it takes for peak titers of antibodies to individual EBV proteins to develop and whether there is a correlation.
between severity of disease and specific EBV protein antibody titers.

MATERIALS AND METHODS

Subjects. Sera were obtained from a prospective study of 31 students at the University of Minnesota from 2006 to 2011 after students gave informed consent (2). The study was approved by the Research Subjects Protection Program of the University of Minnesota. Blood was obtained at least every 8 weeks during the school year and during school breaks if the students remained in the area. The onset of illness in symptomatic subjects was defined as the first day that the subjects reported any symptom consistent with infectious mononucleosis. Severity-of-illness (SOI) scores were determined as described previously (7) using a scale evaluating physical activity and symptom and/or pain intensity that ranged from 0 (asymptomatic) to 6 (bedridden).

EBV antibody assays. (i) EBV fluorescence-activated cell sorter (FACS)-based neutralization assay. Neutralizing antibody titers were determined by a green fluorescent protein (GFP)-based assay that measures the ability of antibody to prevent EBV infection of B cells (8). Human sera were serially diluted in 2-fold steps (from a 1:10 dilution to a 1:1,280 dilution), and 25-μl volumes of the diluted antibody were added to wells of a 96-well plate in duplicate. EBV Akata GFP virus (25 μl) (9) was added to each well and incubated at 37°C for 2 h. The final dilution of the sera was 1:20 to 1:2,560 due to the addition of virus. A 50-μl volume of 1 × 10⁵ Raji cells was added to each well, and the mixture was incubated for 1 day at 37°C. The plate was then centrifuged, the supernatant was removed, and the cells were fixed in 4% paraformaldehyde–phosphate-buffered saline (PBS). The cells were then washed once with PBS.

GFP-expressing cells were quantified using a Guava EasyCyte flow cytometer (Millipore Corporation) and Cytosoft software. The effective concentration of antibody that inhibited infectivity by 50% (IC₅₀) based on reduction of number of GFP-positive cells was calculated using nonlinear regression analysis and GraphPad Prism software. The level of neutralizing antibody was considered below the limit of detection and was assigned an artificial titer of 3.32 log₂ for statistical purposes when the software program failed to fit the results to an appropriate regression curve.

(ii) Enzyme-linked immunosorbent assay (ELISA) for EBV gp350 antibody. Anti-gp350 antibody titers were determined by coating 96-well plates overnight at 4°C with recombinant human gp350 (10) diluted to 0.24 μg/ml in PBS. The next day, the plates were washed extensively with PBS containing 0.05% Tween (PBST) before blocking by addition of Superblock containing 10% Superblock, added to washed plates following blocking, and incubated for 1 h at room temperature. Plates were washed with PBST and then incubated with a 1:7,500 dilution of rabbit anti-human IgG–horseradish peroxidase (IgG-HRP) (Dako catalog no. P0214) and incubated for 1 h at room temperature. Plates were washed with PBST and developed by addition of TMB (3,3′,5,5′-tetramethylbenzidine) for 10 min before the reaction was stopped by addition of 1 N HCl, and the plates were read for absorbance (optical density at 450 nm [OD₄₅₀]) on an Envision system. Data were analyzed using SoftmaxPro software to determine the level of EBV protein-specific antibody in the sample. Light unit values for each antibody were corrected by subtraction of the buffer control value and standardized by dividing the value obtained with control EBV-negative serum. The median fold change (ratio of test sample to EBV-negative serum) was plotted. The cutoff value was determined by calculating the mean plus 2 times the standard deviation (SD) of the fold difference of the known EBV-negative serum. The cutoff values for BZLF1, BMRF1, BHRF1, BLRF2, BFRF3, gp350, gp42, and EBNA1 were 3.97, 1.95, 1.85, 4.85, 2.74, 2.42, 2.80, and 1.72, respectively.

Statistical analyses. The mean or median of neutralization titers of three experiments was used for analysis. The severity-of-illness scores were dichotomized as either ≤2 or ≥3, and the times of onset of symptoms were also dichotomized as ≤100 days versus >100 days. A linear mixed-effect model was used to assess the association between the dichotomized score and neutralization titers. In this model, the neutralization titers were modeled as a linear function of the dichotomized SOI and time values for each patient. The Wilcoxon test was used to compare the differences between groups. The Spearman’s correlation coefficient statistic was used to test the association between two variables.

RESULTS

EBV neutralizing antibody titers increase over the course of several months after primary infection. We first determined EBV neutralizing titers in 4 to 12 serum samples from each of 31 university students collected from as early as 360 days before to as late as 3.7 years after the onset of IM. While the incubation period for IM is 4 to 6 weeks, neutralizing antibody was detected in only 16.1% (5/31) of serum samples before the onset of symptoms. In these subjects, neutralization antibody was detected a mean of 38 days before the onset of symptoms (range, 2 to 62). The median peak in serum neutralizing antibody levels occurred 179 days after the onset of symptoms, and the mean peak was at 191 days (Fig. 1).

We compared the median neutralizing titers determined at between 0 and 100 days after the onset of symptoms to the median titers determined after 100 days from the onset of symptoms. The median neutralizing titer from day 0 to 100 days after the onset of symptoms was 18.0 (mean, 56.4; range, 5 to 492), and the median titer after 100 days from the onset of symptoms was 60.7 (mean, 72.5; range, 5 to 349), indicating that it takes several months for neutralizing titers to reach their maximum values.
The EBV-specific neutralizing antibody response does not correlate with disease severity. To determine whether EBV neutralizing antibody titers correlate with disease severity, the peak neutralizing antibody titer for each subject was compared with the subject’s severity-of-illness score, which was the sum of the scores corresponding to physical activity limitation and symptom/pain intensity, which ranged from 0 (asymptomatic) to 6 (bedridden) (7). The median peak EBV neutralizing titer in 15 subjects with severity-of-illness scores of 1 to 3 was 84.8 (mean, 116; range, 9.8 to 349) (Fig. 2A), and the median peak in 16 subjects with severity-of-illness scores of 4 to 6 was 128.6 (mean, 170; range, 14 to 492) (Fig. 2B). The median number of days to peak EBV neutralizing titers in subjects with severity-of-illness scores of 1 to 3 was 198 and in those with severity-of-illness scores of 4 to 6 was 161.

We found no correlation between the median peak EBV neutralizing titer and the severity-of-illness scores (Fig. 3A and B).

EBV gp350 ELISA antibody titers increase over the course of several months after primary infection. EBV gp350 is the primary target of neutralizing antibody to EBV and is the envelope protein that is most highly expressed on virions and in infected cells. We measured EBV gp350 antibody levels by ELISA in each of the subjects over time. ELISA antibody to EBV gp350 was present a median of 29 days before the onset of symptoms (range, 4 to 65 days). The median peak in gp350 ELISA antibodies was seen 333 days after the onset of symptoms, and the mean peak was at 390 days (Fig. 1). The median peak EBV ELISA gp350 titer in 15 subjects with severity-of-illness scores of 1 to 3 was 77.07 and in 16 subjects with severity-of-illness scores of 4 to 6 was 56.15 (Fig. 3C and D; see also Fig. S1 in the supplemental material). These differences were not significant (SOI = 1 to 3 versus SOI = 4 to 6, P = 0.953). The median number of days to peak gp350 ELISA antibody titers in 15 subjects with severity-of-illness scores of 1 to 3 was 333 and in 16 with severity-of-illness scores of 4 to 6 was 300.

Kinetics of antibody responses to other EBV proteins using an immunoprecipitation assay. We next determined the level of antibody to 8 EBV proteins using a luciferase immunoprecipitation system (LIPS) assay in 14 subjects from whom sufficient sera were available for testing. The LIPS test is more likely to measure levels of antibody to the native form of the protein than the ELISA. Results of the gp350 LIPS assay have been shown to have a strong
correlation with the neutralizing antibody titer in human sera (8). Levels of antibody to EBV immediate early protein BZLF1 (a transcriptional activator), early proteins BMRF1 (DNA polymerase processivity factor) and BHRF1 (homolog of Bcl-2), late proteins gp350 (glycoprotein for virus attachment and entry), gp42 (glycoprotein for B cell infection), BLRF2 (p23 capsid protein), and BHRF1 (capsid protein), and latency-associated protein EBNA1 were determined (Fig. 4). We found no correlation between the viral load and EBV neutralization titers or antibodies to individual viral proteins determined by the LIPS assay.

Antibodies to EBV proteins could be divided into three groups based on the time required to reach the peak titer. LIPS antibody responses to the first group, EBV gp350 and the EBNA1 latency-associated protein, took longer to peak than the responses to any of the other EBV proteins that were tested. The median number of days after the onset of IM symptoms to peak antibody titers for gp350 and EBNA1 was 911 and 635, respectively, indicating that it took about 1.5 to 2.5 years for peak antibody titers to develop to these proteins (Fig. 1). Antibody responses to a second group of EBV proteins were directed to BHRF1, BMRF3, and gp42 and required a little over a year—medians of 472, 444, and 420 days, respectively—to reach peak titers. Antibodies responses to a third group of viral proteins, BMRF1, BZLF1, and BLRF2, took nearly one-half year—medians of 209, 180, and 123 days, respectively—to reach peak titers. While the lengths of time to peak titers of antibody were different for individual proteins, EBNA1 and the two glycoproteins tended to take longer to reach peak titers.

The same clustering of antibodies to EBV proteins observed to correspond to lengths of time to peak titers was found when median peak levels of antibody titers were measured. Median peak antibody titers to gp350 and EBNA1 were increased 157.1- and 89.8-fold relative to control EBV-negative serum results, respectively (Fig. 5). Median peak levels of antibody to gp42, BMRF3, and BHRF1 were increased 40.6-, 12.8-, and 18.8-fold relative to the negative-control serum results, respectively. Median peak titers of antibody to EBV proteins BLRF2, BZLF1, and BMRF1 were higher than the levels seen with negative-control serum by 7.4-, 9.1-, and 2.3-fold. There was no correlation between the median peak antibody titers to EBV proteins determined by the LIPS assay and the severity-of-illness score (Fig. 5; see also Fig. S2 to S9 in the supplemental material). However, the correlation between median peak titers and median time to peak titers was significant (Spearman’s correlation coefficient $r = 0.86$, $P = 0.025$) (Fig. 6).

### DISCUSSION

In this study, we followed the kinetics of neutralizing antibodies and virus-specific antibodies in university students after primary infection with EBV. Other studies have followed only very small groups of individual subjects with primary EBV infection over time, and specific EBV protein or neutralizing antibody levels were not measured (12–15). One study measured neutralizing antibodies in 128 subjects at various times after IM, and, while some subjects contributed two or more serum samples, the level of neutralizing antibody in individual subjects was not plotted over time (3). In the latter study, the level of neutralizing antibody peaked at 3 to 4 months after IM; in contrast, in our study, the level of neutralizing antibody peaked much later, at a median of 6 months. The difference between these two studies may have been due to the short period of time that each patient was followed in the study by Hewetson and colleagues, the difference in neutralization assays used, or the design of our study, which included those with atypical symptoms. Recently, a more limited study showed that neutralizing and gp350 antibody responses were delayed after primary infection and the authors attributed this to a reduced number of memory B cells (16).

We found no association between the severity of illness and the level of specific EBV protein or neutralizing antibody levels. It is possible that a correlation might be observed with a larger sample pool. Using the original patient cohort, the severity-of-illness score was found to correlate with the levels of EBV DNA, CD8 T cells, and NK cells in the blood (2); however, in the subset of patients whose serology we evaluated by LIPS assay, we did not detect a correlation of EBV DNA with titers of antibodies to viral proteins. A study of 8 patients with prolonged illness after IM found that neither levels of EBV DNA in the blood nor levels of EBV antibodies correlated with severity of disease or duration of
illness (17). Another study of 18 patients with IM found a correlation between the severity of disease and the cellular immune response to EBV proteins but not to levels of antibody to VCA or early antigens (18). Based on trials of an EBV subunit vaccine which induced antibody (but not cellular) responses to EBV in most participants and reduced the rate of infectious mononucleosis but not EBV infection (19, 20), antibodies likely have a role in reducing symptoms of EBV infection. It is possible that other activities of antibodies that we did not measure, such as antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity, might also control the severity of EBV disease. However, cellular immune responses to EBV are thought to be more important to control the severity of virus disease than antibody responses, and persons with defects in T cell immunity, including transplant recipients and patients with congenital immunodeficiencies, may develop severe EBV disease (reviewed in references 21 and 22).

Of the antibodies to 8 viral proteins that we studied, antibodies to EBNA1 and gp350 showed the highest levels after EBV infection compared to the levels in seronegative controls (>50-fold). Levels of these two antibodies measured by immunoprecipitation assay generally increased over time after infection and often did not reach peak titers until about 2 years after the onset of IM symptoms. EBV gp350 is the most abundant virus envelope protein in virions and binds to its receptor CD21 on B cells, initiating virus attachment and entry. The median time to peak titers for EBV gp350 measured by the LIPS assay was much longer (911 days) than the time measured by the ELISA (333 days). In a recent study, we found that mice vaccinated with different gp350 immunogens developed high gp350 ELISA titers relatively early after vaccination but that high gp350 LIPS titers took much longer to develop and required additional doses of immunogen (23). The gp350 LIPS test, an immunoprecipitation assay, is more likely to detect antibody primarily to the native form of the protein, while the ELISA may measure antibodies to both denatured and native forms of the protein (24). In another study, we found that levels of antibody to EBV gp350 measured by an immunoprecipitation assay correlated very closely with levels of neutralizing antibody in human sera (8). Antibody to gp350 is the major component of EBV neutralizing antibody in human sera (25, 26). Antibody to gp42 blocks entry of EBV into B cells, and there is a more modest correlation of gp42 antibodies with the neutralizing antibody response in humans (8). Peak titers of gp42 antibody occurred a median of 420 days after the onset of IM symptoms. Thus, there is a long delay between the onset of IM and peak titers of antibodies important for EBV infection of B cells.
EBV EBNA1 is the only viral protein expressed in all forms of EBV latency and is required for viral DNA episome maintenance in latently infected cells. We found that antibody to EBV EBNA1 appeared later in the sera than antibody to other EBV latency proteins, including EBNA2, EBNA3, and EBNA-LP (5, 27). There is a similarly delayed appearance of CD4 T cell responses to EBNA1 in the blood after the onset of IM compared with EBNA2 and other EBV lytic proteins (28). This delay may be due to the fact that, unlike EBV lytic proteins and the other EBNAs, EBNA1 is not normally released from EBV-infected cells or that EBNA1 inhibits its own translation and degradation, resulting in reduced presentation to T cells. Previous studies have shown that patients with severe EBV diseases, such as chronic active EBV (5) or EBV-positive lymphomas (29), have impaired antibody responses to EBNA1 or altered IgG subclass responses to EBNA1, respectively. Thus, appropriate antibody responses to EBNA1 are associated with control of EBV infection.

In summary, using sera from a cohort of university students with primary EBV infection followed for a median of 3 years, we have found that levels of EBV neutralizing antibody, as well as levels of antibody to EBV gp350 and EBNA1, increase over time after infection and take up to about 2 years or more to reach peak levels, while levels of antibodies to other viral proteins peaked at 6 to 16 months after infection. While prior studies have shown that levels of EBV DNA, CD8 cells, and NK cells in the blood correlated with the severity of IM, we did not observe a correlation between the severity of disease and specific EBV protein antibody titers.

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