

Epstein-Barr Virus Antibodies in Whole Blood Spots: A Minimally Invasive Method for Assessing an Aspect of Cell-Mediated Immunity

THOMAS W. McDADE, PhD, JOY F. STALLINGS, PhD, MPH, ADRIAN ANGOLD, MRCPsych, E. JANE COSTELLO, PhD, MARY BURLESON, PhD, JOHN T. CACIOPPO, PhD, RONALD GLASER, PhD, AND CAROL M. WORTHMAN, PhD

Objective: Study 1: Introduce and validate a method for measuring EBV p18-VCA antibodies in whole blood spots to provide a minimally invasive marker of cell-mediated immune function. Study 2: Apply this method to a large community-based study of psychopathology in children and adolescents. **Methods:** The EBV antibody method was evaluated through analysis of precision, reliability, stability, and comparisons with plasma and indirect immunofluorescence methods. The effects of life events on p18-VCA antibody level were considered in a subsample of 9, 11, and 13 year-old children participating in the Great Smoky Mountains Study in North Carolina. The subsample was stratified by age, sex, and degree of overall life strain. **Results:** Dried blood spots provided a convenient, sensitive, precise, and reliable method for measuring EBV p18-VCA antibody titer. Life events were positively associated with p18-VCA antibodies in girls but not in boys. **Conclusions:** The validity of the blood spot EBV p18-VCA antibody assay, as well as the ease of sample collection, storage, and transportation, may provide an opportunity for psychoneuroimmunology to explore a wider range of stress models in larger, community-based studies. **Key words:** psychoneuroimmunology, cell-mediated immunity, stress, methods, life events, sex difference.

EBV = Epstein-Barr virus; VCA = viral capsid antigen; IgG = immunoglobulin G; IF = indirect immunofluorescence; ELISA = enzyme-linked immunosorbent assay; CV = coefficient of variation; GSMS = Great Smoky Mountains Study; CAPA = Child and Adolescent Psychiatric Assessment.

INTRODUCTION

Psychoneuroimmunology continues to unravel the complex interconnections between psychosocial stress and immune function, emphasizing the relevance of social ecology to investigations of human health. However, certain methodological limitations have constrained these investigations. Venipuncture and catheterization are relatively invasive sampling procedures that require the skills of trained medical professionals. Once collected, blood samples must be promptly centrifuged, separated, and quick-frozen or immediately assayed, which requires ready access to laboratory facilities. These factors have limited the range of participants that can be recruited for most psychoneuroim-

munological studies and prevented the exploration of stress-immunity relationships in larger epidemiological, community-based studies.

In this article, we introduce and validate a minimally invasive, indirect method for measuring one aspect of cell-mediated immune function: antibodies against EBV in whole blood spot samples. Previous work has shown that EBV antibody level is a valid marker of cell-mediated immunity that responds to a range of psychosocial stressors (1–6). While these studies have used serum or plasma from venipuncture, we have validated an EBV-VCA antibody assay for whole blood samples from finger pricks. The method minimizes the inconvenience and burden imposed on participants by blood sampling and eases the requirements for sample collection, storage, and transportation before analysis. Such features allow for the collection of samples away from the research laboratory or clinic and provide access to individuals for whom venipuncture may be problematic, such as children or the elderly. In this article, we first present this method in detail, then demonstrate its utility for psychoneuroimmunology with pilot data from a large, community-based study of traumatic life events and immune function in children and adolescents from North Carolina.

STUDY 1: VALIDATION OF AN INDIRECT METHOD

Methods: Sample Collection.

The requirements for blood spot sample collection, storage, and transportation are minimal. Each participant's finger is cleaned with alcohol, then pricked with a sterile, disposable microlancet that is widely available for use by diabetic patients (Microtainer, Franklin Lakes, NJ). Up to five drops of whole blood are collected on standardized filter paper commonly

From the Department of Anthropology (T.W.McD.), Northwestern University, Evanston, IL; Department of Anthropology (J.F.S., C.M.W.), Emory University, Atlanta, GA; Center for Developmental Epidemiology (A.A., E.J.C.), Duke University Medical Center, Durham, NC; Department of Social and Behavioral Sciences (M.B.), Arizona State University West, Phoenix, AZ; Department of Psychology (J.T.C.), University of Chicago, Chicago, IL; and Department of Molecular Virology, Immunology and Medical Genetics (R.G.), Ohio State University Medical Center, Columbus, OH.

Address reprint requests to: Thomas W. McDade, PhD, Northwestern University, Department of Anthropology, 1810 Hinman Ave, Evanston, IL 60208. Email: t-mcdade@nwu.edu

Received for publication February 22, 1999; revision received November 17, 1999.

EBV ANTIBODIES IN WHOLE BLOOD SPOTS

used for neonatal screening (Schleicher and Schuell #903, Keene, NH). One drop of blood (approximately 50 μL) is sufficient because the assay requires only one 2.5-mm disk of dried blood for duplicate analysis. Samples are dried overnight and can remain at room temperature for up to 8 weeks before analysis or freezer storage (see stability analysis below). In comparison with venipuncture, this is a relatively painless and noninvasive procedure and does not require centrifugation or immediate freezing. Dried blood spot samples are not considered biohazardous and can be shipped without special documentation from the site of collection to the laboratory. Samples do not need to be kept refrigerated or frozen during transport. Before shipment, samples should be sealed in plastic bags or plastic containers to ensure protection from moisture.

Methods: EBV Antibody Assay

Assay Principle. The method used here is an ELISA for measuring p18-VCA antibodies in whole blood spots. Most studies have used IF to measure EBV antibody levels, in which substrates expressing EBV antigens are incubated with serial dilutions of subject sera. Using IF, EBV antibody titers are determined by the final dilution at which IF-positive cells are no longer detectable. ELISA provides another measure of EBV antibody titer, correlates well with IF (see below), and has been shown to be a more sensitive, objective, and less labor-intensive method (6, 7). Although previous work has demonstrated that dried blood spots are a convenient and accurate medium for measuring specific antibodies (8, 9) as well as hormones such as cortisol, testosterone, estradiol, and gonadotropins (10, 11), this study is the first to use a blood spot method to measure EBV antibodies.

The method is an adaptation of a commercially available kit for measuring p18-VCA IgG antibodies in serum (No. 7590, DiaSorin Corporation, Stillwater, MN). The assay measures antibodies against one VCA protein, the p18 polypeptide, which is a marker protein containing immunodominant epitopes of the viral capsid antigen complex. Antigen-antibody complexes form between p18-VCA IgG antibodies present in the sample and synthetic p18 peptide bound to the surface of microtiter wells. Horseradish peroxidase-labeled antihuman IgG in the presence of a chromogen substrate reacts with the antigen-antibody complex resulting in color development. The concentration of p18-VCA IgG antibody is directly related to the absorbance of the solution measured at 450 nm.

Standards and Controls. The manufacturer provides four levels of p18-VCA IgG standard in stabilized hu-

man sera. Controls consist of three levels of manufacturer-supplied control sera and four levels of blood spot controls made from whole blood pools spotted onto filter paper and stored at -23°C . Standard concentrations are provided by the manufacturer and are used to convert absorbance values to standard ELISA scores. Blood spot control ranges are derived from values obtained over multiple assays.

Elution of Whole Blood Spots. The day before an assay is to be performed, blood spot samples and controls are removed from the freezer, and a small hole punch (available from local office supply stores) is used to punch out a 2.5-mm diameter disk of whole blood. The disk is transferred with tweezers to a labeled 12×75 glass tube, and 250 μL of diluent buffer (supplied with kit) is added. The sample is incubated overnight at room temperature. The following day, the assay is performed as per the kit instructions, with 100 μL of the blood spot eluate pipetted directly into microtiter wells in duplicate, along with 100 μL of each standard and diluted control sera.

Assay Validation

Plasma/Blood Spot Correlation. Complex antigen-antibody binding dynamics preclude direct evaluation of linearity and accuracy by manipulating EBV antibody concentration. Therefore, blood spot antibody methods rely on comparisons with plasma values for validation (12, 13). Matched plasma and whole blood samples were obtained from 40 adult subjects. From each whole blood sample (EDTA-anticoagulated), a portion of whole blood was spotted onto filter paper, and another portion was centrifuged and the plasma withdrawn. Plasma p18-VCA antibody values were obtained following kit instructions and compared with matched blood spot values. Correlation between blood spot and plasma values is linear and high (Pearson correlation $R = 0.97$) (Figure 1).

Seropositivity Determination. The accurate determination of EBV serostatus is critical because the model linking stress, suppressed cell-mediated immune function, and subsequent increase in EBV antibody titer does not apply to seronegative individuals who are not latently infected with EBV. Analyses must therefore focus exclusively on seropositive individuals, and conclusions drawn from statistical analyses can only be applied to the population of seropositives. Fortunately, the ubiquity of EBV exposure worldwide—especially in the developing world—ensures that 80% to 100% of adults will be seropositive (14). However, studies of children, and research conducted among populations with low burdens of infectious disease, must pay particular attention to EBV serostatus.

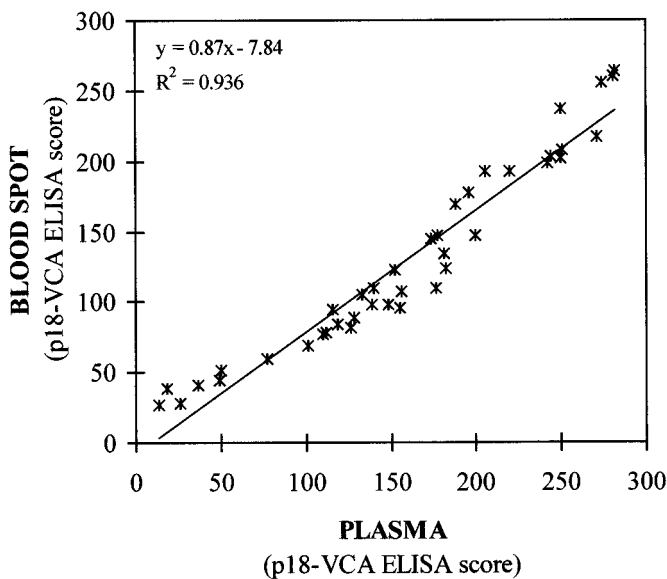


Fig. 1. Correlation between plasma and blood spot EBV p18-VCA antibody titer.

According to kit specifications, a serum value ≥ 20 ELISA units indicates the presence of p18-VCA antibodies. A serum value < 20 ELISA units indicates non-detectable levels of p18-VCA antibodies, and these individuals are thus presumed to be seronegative for EBV. This cutoff was established by the kit manufacturer through analysis of samples from individuals before and after becoming ill with infectious mononucleosis. To establish a seropositivity cutoff value for the blood spot method, 19 individuals with low titers of EBV antibodies were identified, and matched plasma and whole blood spot samples were run together in one assay. According to this analysis, the plasma cutoff of 20 corresponds to a blood spot cutoff of 18. If the plasma cutoff of 20 is considered to be the standard, then the blood spot cutoff of 18 resulted in one false-negative and one false-positive. Increasing the blood spot cutoff to 20 resulted in two false-negatives and zero false-positives, thereby increasing specificity at a cost to sensitivity. Further increases in the blood spot cutoff resulted in more false-negatives. Although the number of individuals in this analysis is small, it provides justification for a blood spot seropositivity cutoff of 18 or 20. We have elected to use the more conservative cutoff of 20 because this corresponds to the kit specifications and limits the number of false-positives. As a consequence of this cutoff, a small number of false-negative individuals with low levels of EBV p18-VCA antibodies may be eliminated from analysis.

Comparison With IF. Indirect immunofluorescence is the current gold standard for assessing EBV antibody

titers and has been used in numerous studies linking psychosocial stress to immune function (1, 4, 15). To compare the blood spot ELISA EBV-VCA method with plasma IF, 111 undergraduate students (mean age, 19.2 years; range, 18–24) were recruited at Ohio State University. Blood samples were collected with a 20-gauge indwelling angio-catheter, which was inserted into an antecubital vein on each subject's dominant arm. At least 30 minutes after insertion, a 20-mL blood sample was drawn into a syringe, and blood spots were made for each participant by placing five drops of whole blood onto filter paper. Blood spots were allowed to dry at room temperature overnight and were then frozen until delivery to the Laboratory for Comparative Human Biology at Emory University. Blood spot samples were stored at -23°C until ELISA analysis. The remaining blood sample was injected into heparinized collection tubes and centrifuged, and the plasma was stored at -70°C at Ohio State until IF analysis.

The indirect IF test was used to measure EBV-VCA IgG antibody titers in plasma as previously described (16). Briefly, acetone-fixed HR-1 cells (a cell line latently infected with EBV) prepared on glass coverslips were adsorbed with two-fold dilutions of plasma in phosphate-buffered saline. Approximately 5% of these cells were early antigen/VCA positive by IF. The highest dilution of plasma that resulted in detection of at least 1% IF positive cells was considered the end point. All slides were blind coded and read by examining 300 to 400 cells.

Before statistical analysis, ELISA and IF results were log-transformed to normalize the distributions. There was a significant positive relationship between the two measures (Pearson $R = 0.77$; $p < .01$) (Figure 2). The strength of this relationship is consistent with previous work comparing IF and ELISA methods for serum EBV antibody titers (7). A perfect correlation between IF and ELISA was not expected because our blood spot ELISA method only detects antibodies against p-18, the immunodominant VCA epitope. In contrast, the less-specific IF method detects antibodies to multiple EBV proteins. Individual differences in the heterogeneity of antibody responses to VCA can thus result in different EBV antibody levels across these methods.

Precision and Reliability. Within-assay and between-assay %CV were calculated with multiple determinations of four blood spot controls representing the entire assay range. Precision was assessed with 10 determinations of each control run within a single assay. Reliability was assessed by running each control in duplicate across 12 assays performed on different days. As shown in Table 1, results indicate a high degree of assay precision and reliability, with the high-

EBV ANTIBODIES IN WHOLE BLOOD SPOTS

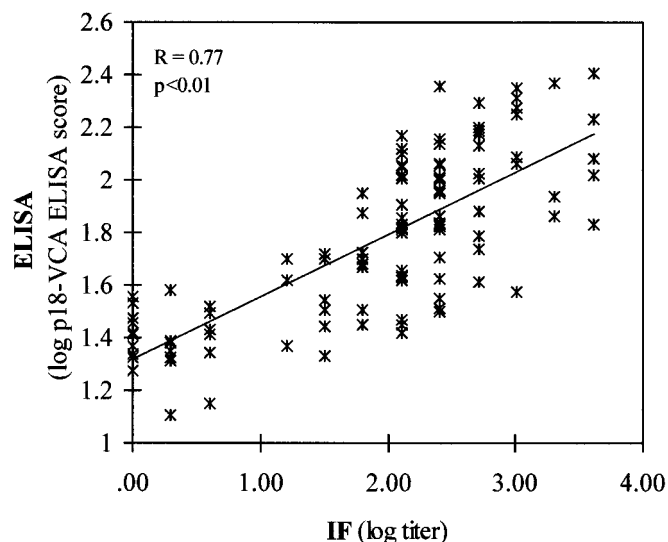


Fig. 2. Correlation between blood spot ELISA method and plasma IF.

TABLE 1. EBV p18-VCA Antibody Assay Precision (Within-Assay CV) and Reliability (Between-Assay CV)^a

Control Level	Within-Assay (N = 10 Determinations)		Between-Assay (N = 12 Runs)	
	Mean ± SD	%CV	Mean ± SD	%CV
Low	29.4 ± 2.7	9.2	30.6 ± 4.3	14.1
Mid-low	111.4 ± 3.7	3.3	100.2 ± 5.0	5.0
Mid-high	212.1 ± 11.1	5.3	206.3 ± 8.8	4.3
High	271.7 ± 12.1	4.5	278.3 ± 19.1	6.9

^a Mean p18-VCA antibody titers are presented in standard ELISA scores.

est %CV values found at the low end of the assay range.

Linearity. Samples reading beyond the assay range must be diluted at the end point. To evaluate linearity, 10 mid-high control blood spot samples were read and then diluted 1:2 with stop solution. Mean recovery values were 102.8% of expected values, ranging from 100.8% to 103.1%.

Cross-Reactivity. This method is reported by the kit manufacturer not to be cross-reactive with Herpes Simplex virus I and II, Cytomegalovirus, Varicella Zoster virus, Toxoplasma, and Rheumatoid factor.

Stability. The stability of p18-VCA antibodies in blood spot samples was determined over an 8-week period in which blood spot cards made from a single venipuncture were exposed to one of three temperature conditions (4°C, room temperature, and 37°C) for varying lengths of time up to 8 weeks. A sample at the high end of the assay range was used to maximize the likelihood of detecting any deterioration. In addition,

the potential impact of humidity was considered by sealing one set of cards in plastic bags with approximately 30 g of desiccant (CaSO₄). The other set of cards was not exposed to desiccant. Samples were considered to be stable as long as values remained within a 10% CV range of the initial values. Results for the samples with desiccant are presented in Figure 3, although no difference in the pattern of deterioration was found with or without desiccant. Samples remained stable for at least 8 weeks at 4°C and room temperature, but began to deteriorate after 1 week at 37°C.

To evaluate stability in the freezer, four blood spot controls were made and promptly assayed to assess precision (Table 1). After 2 years of storage at -23°C, the controls were assayed again (10 determinations per control). Mean p18-VCA antibody titers were comparable, showing no evidence of deterioration after 2 years of freezer storage (Low control (mean ± SD): 23.2 ± 2.6; Mid-low: 104.8 ± 4.3; Mid-high: 214.9 ± 10.1; High: 280.3 ± 7.6).

Overall, these results indicate a high degree of EBV antibody stability in whole blood spots. Although prompt freezer storage is always desirable, samples can be collected in the field and stored at room temperature for up to 2 months. In settings where temperatures are likely to exceed 22°C to 23°C, attempts should be made to protect samples from excessive heat.

Freeze/Thaw. The stability of p18-VCA antibodies to repeated cycles of freezing and thawing was evaluated for four blood spot controls that represented the entire assay range. Blood spot cards were removed from the freezer each afternoon, left at room temperature for 2 to 3 hours, and returned to the freezer. This

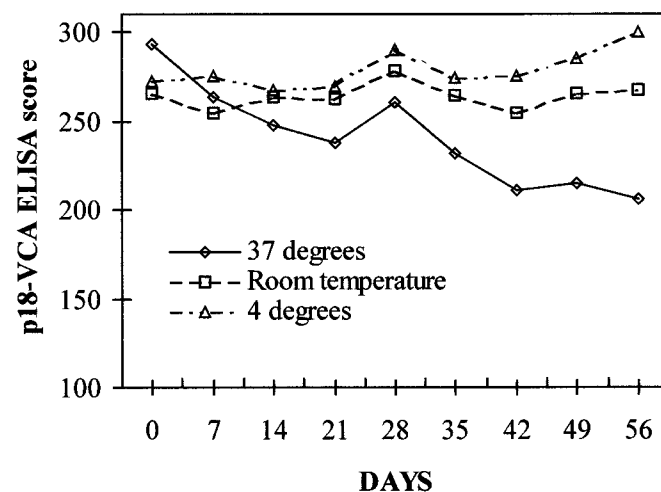


Fig. 3. Stability of EBV p18-VCA antibodies in whole blood spots over 8 weeks at 37°C, room temperature, and 4°C.

protocol was repeated for six consecutive days. Samples were considered stable if they remained within a 10% CV range of the sample that remained frozen 24 hours after preparation. No sample deterioration was detected for up to six freeze/thaw cycles.

STUDY 2: STRESS AND EBV ANTIBODIES IN NORTH CAROLINA CHILDREN AND ADOLESCENTS

Psychoneuroimmunology has long recognized the importance of developmental aspects of stress and immunity (17); but to date, few studies have addressed this subject. Small, clinic-based studies have explored the immunological effects of major depressive disorder and conduct disorder (18–21), whereas studies by Boyce and colleagues (22, 23) focused on kindergarten entry as a significant stressor of early childhood. These studies suggest important links between psychosocial experience and immune function in childhood and adolescence, but are limited by their small sample sizes or clinical design. This pilot study evaluated the utility of our blood spot EBV antibody method in a large community-based sample of children and adolescents and suggests that developmental and contextual variables may be important moderators of stress-immune function relationships in this population.

Methods

Participants. This study focused on a subsample of 9-, 11-, and 13-year-old children participating in the ongoing Great Smoky Mountains Study of adolescent psychopathology and service use. This large community-based study relates symptoms of child psychopathology to comprehensive measures of life strain, socioeconomic status, family environment, and morphological and endocrine measures of pubertal status.

Methodological details for the GSMS are presented elsewhere (24). Briefly, a multistep sampling procedure was used to select among the 12,000 9-, 11-, and 13-year-old children listed in the public school database of the southern Appalachian mountain region of North Carolina. Within each age category, children were selected on an equiprobability basis to generate a total screening sample of 4500 children. A screening questionnaire was administered to parents of 3896 children to identify children with a high probability of psychiatric symptoms. All children with scores above a predefined cutoff point were recruited for the main study. A 1 in 10 random sample of the remainder was also recruited. Of the 1346 children recruited for the study, 1071 (79.6%) were enrolled. Children and their parents were interviewed as close as possible to the

birthday on which children became 9, 11, or 13 years of age.

In this population, family dysfunction, negative life events, and poverty are unsurprisingly and strongly associated with psychiatric symptoms (24). Previous findings have reported the development of a significant sex difference in the relationship between stressful life circumstances and symptoms of depression. For girls, advancement through puberty is associated with a strengthening of the relationship between life strains and depression, whereas puberty acts as a protective factor for boys, actually eliminating the correlation between hardship and depression (25).

Accordingly, in this study we sought to evaluate the immunological consequences of stressful life circumstances and hypothesized that a similar pattern of sex difference would emerge in puberty. Therefore, we selected a subsample of children stratified by sex (male/female), age (9, 11, and 13 years), and degree of overall life strain (low/high), yielding 12 groups. Life strain scores were constructed from the following: welfare dependency, parental unemployment, low parental education, family size (four or more children), the presence of domestic violence, parental drug use, parental arrest, maternal depression, and diagnosed psychopathology among family members. Scores ranged from 0 to 32 in the main sample, from which two subsets of participants were drawn: individuals with scores <10 comprised the low-strain group, whereas those with scores >14 comprised the high-strain group. An attempt was made to assign 30 individuals to each of the 12 groups, but this was not possible, largely due to higher rates of refusal to give blood among girls. No group had fewer than 16 individuals, and the final sample size for this pilot study was 256, representing 23.9% of the overall GSMS sample.

Measures of Psychosocial Stressors. The CAPA protocol was used in parent and child interviews to gather information on psychopathology and life circumstances and experiences (26). This structured protocol elicits information regarding symptoms for a wide range of diagnoses, functional impairment, and the quality of family relationships.

In addition to overall life strain, we considered the number of traumatic life events, defined as death of a close family member and/or friend, sexual and/or physical abuse, and exposure to other traumatic experiences (eg, extreme violence or accident) endured over a child's lifetime. Children had experienced on average 1.0 traumatic life events ($SD = 1.2$), with a range of 0 to 6. Children in the high-strain group experienced significantly more life events than children in the low-strain group (1.42 vs. 0.65, $p < .001$). Trau-

EBV ANTIBODIES IN WHOLE BLOOD SPOTS

matic life events were not included in our summary measure of life strain.

Sample Collection. Blood spot samples were procured as described above. At least two drops of capillary whole blood were collected on filter paper, dried overnight at room temperature, and placed in a refrigerator for no more than 3 weeks before shipment to the laboratory for storage at -23°C until analysis. The ELISA method described above was used to determine the EBV p18-VCA antibody level for each individual.

Statistical Analysis. Statistical analyses were performed for all EBV-positive individuals using Statistical Analysis Software (Version 6.12, SAS Institute Inc., Cary, NC). Before analyses, EBV antibody scores were logarithmically transformed to normalize the distribution. We set $\alpha = 0.05$ as our criterion for statistical significance.

Results

Of the initial 256 study participants sampled, 80.1% were seropositive for EBV according to ELISA detection of p-18 VCA antibodies, yielding a final sample of 205 for our analyses. Table 2 presents the untransformed mean EBV antibody levels according to our sampling strategy and the distribution of participants across the sampled groups. Boys were overrepresented in our sample because girls were more likely to refuse to give blood, but boys were no more likely than girls to be seropositive ($\chi^2 = 0.22$, NS).

Previous work in this population demonstrated a sex-differentiated relationship between stress and symptoms of depression (25). Therefore, initial analyses were conducted separately for boys and girls. To evaluate the effects of our sampling variables, age and degree of life strain were included in ANCOVA models, with log-transformed p18-VCA antibody titer as the dependent variable. EBV p18-VCA antibody titers were higher for both boys and girls in the high-strain vs. low-strain groups, although these differences failed

to reach statistical significance (boys (mean \pm SE): low strain = 81.0 ± 7.5 ELISA units, high strain = 94.0 ± 7.3 ELISA units, $p = .12$; girls: low strain = 69.6 ± 7.5 ELISA units, high strain = 88.7 ± 7.6 ELISA units, $p = .18$). The interaction between age and life strain did not approach significance for boys or girls.

Although neither age nor degree of life strain were significantly related to p18-VCA antibodies, we included these variables in subsequent regression analyses because they comprised our sampling criteria. The addition of traumatic life events resulted in a significant model for girls, with traumatic life events positively related to p18-VCA antibody titer, whereas the sampling variables, including life strain, were non-significant (Table 3). The partial correlation coefficient between number of life events and p18-VCA antibody titer—indicating the strength of the relationship between these two variables, controlling for the effects of age and life strain—was $R = 0.31$ in girls ($p < .01$). In contrast, life events were not significantly related to p18-VCA for boys (partial $R = 0.007$, NS).

The addition of interaction terms did not improve the fit of these models, with one exception: the interaction between life events and life strain approached significance for girls ($p = .096$). Number of life events remained as a significant independent predictor of p18-VCA when this interaction term was included in the model. Girls exhibited a strong positive relationship between life events and p18-VCA antibody titer under conditions of high strain, (partial $R = 0.45$, $N = 44$, $p < .01$), but no relationship under low life strain (partial $R = 0.05$, $N = 45$, NS). No such effect was found for boys (Figure 4). In other words, under conditions of high life strain, girls with one or more traumatic life events had antibody titers that were approx-

TABLE 3. Results From Multiple Linear Regression Model of Log-Transformed EBV p18-VCA Antibody Titer Including Sampling Variables and Traumatic Life Events^a

	Variable	Parameter Estimate	Type II Partial Correlation
Girls (N = 89)	Age	-0.015	
	Life strain	0.022	
	Life events	0.060**	0.31
	Model $R^2 = 0.12^*$		
Boys (N = 116)	Age	0.006	
	Life strain	0.065	
	Life events	0.017	0.007
	Model $R^2 = 0.026$		

^aAnalyses were performed separately for girls and boys.

* $p < .05$.

** $p < .01$.

TABLE 2. EBV p18-VCA Antibody Titer by Sampling Variables (Age, Gender, and Degree of Life Strain)^a

Age of Subjects (y)	Low Strain		High Strain	
	Girls	Boys	Girls	Boys
9	67.9 \pm 18.8	71.1 \pm 12.1	106.8 \pm 18.1	104.1 \pm 13.4
N	13	15	12	23
11	75.7 \pm 9.5	88.2 \pm 12.4	70.4 \pm 14.5	77.6 \pm 9.9
N	16	22	15	20
13	65.0 \pm 7.1	80.5 \pm 14.1	92.1 \pm 15.3	99.7 \pm 12.9
N	16	19	17	17

^aMean p18-VCA ELISA score \pm SE.

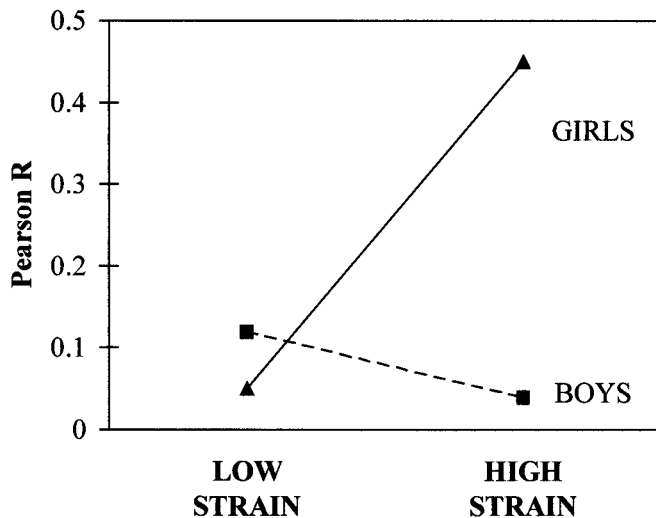


Fig. 4. Relationship between traumatic life events and EBV p18-VCA antibody titer under conditions of low and high life strain. Pearson *R* indicates the strength of the relationship between number of life events and log p18-VCA antibody titer, controlling for age.

imately twice as high as girls with no life events (≥ 1 event, 117.4 ± 16.4 ELISA units; no events, 64.6 ± 9.8 ELISA units). In contrast, there was no difference in antibody titers for girls under conditions of low life strain, regardless of the number of life events (≥ 1 event, 68.2 ± 14.5 ELISA units; no events, 70.5 ± 6.8 ELISA units).

DISCUSSION

The Epstein-Barr virus is an ubiquitous herpesvirus that infects 80% to 90% of adults by the age of 40 (14, 27). Once infected, individuals harbor the virus for life, and adequate cell-mediated immune function is critical for maintaining the virus in a latent state. Immunosuppression allows EBV to reactivate and release viral antigens into circulation, to which a humoral antibody response may emerge (2). As a result, levels of antibodies against EBV antigens provide an indirect measure of cell-mediated immune function.

Studies of stress-induced immunosuppression have reliably demonstrated the utility of the EBV model (28). Academic exam stress in medical students has been linked to increases in EBV antibody titers and concurrent reductions in EBV-specific memory T-cell proliferation and cytotoxic T-cell killing of infected cells (3, 16). Similar results are reported for the chronic stress associated with caring for a family member with Alzheimer's disease (29) or involvement in a poor quality marriage (30, 31). Additionally, loneliness, defensiveness, and anxiety have all been positively associated with EBV antibodies (1, 32). Re-

cently, EBV reactivation has also been associated with the anticipation of space flight by astronauts (33). Conversely, stress management interventions and disclosure of previously repressed trauma are associated with decreases in EBV antibodies (6, 34). These studies validate the EBV model as an indirect measure of stress-induced cell-mediated immune suppression such that higher stress burdens are reflected in higher EBV antibody titers.

Assay validation studies reported here uniformly demonstrate that our blood spot method of EBV antibody determination represents a sensitive, reliable, and stable measure. Results from a pilot study of stress and EBV VCA antibody level in children and adolescents in North Carolina furthermore demonstrate the field utility of our blood spot method. The ease of sample collection, storage, and transportation facilitates the acquisition of samples outside of the confines of the clinic or laboratory.

In addition to minimizing participant burden and maximizing researcher convenience, this method may provide an opportunity for psychoneuroimmunology to explore a wider range of stress models in larger community-based samples. Such an epidemiological approach allows one to recruit larger, more representative samples that can overcome some of the limitations of clinic-based studies. The minimal pain and inconvenience imposed by finger pricks also facilitates more frequent blood sampling and even enables participants to collect samples from themselves at home (35). Field studies of stress and immune function in diverse ecological contexts are also feasible (36). Blood spot methods for gonadal and adrenal steroid hormones have been used successfully in cross-cultural studies of reproductive ecology around the world, including Papua New Guinea, East Africa, Nepal, and North Carolina (11).

Although the results of this study show that the use of a commercially available ELISA kit using one EBV polypeptide can be useful, it is also important to note that measuring differences in antibodies to only one viral peptide may provide aberrant results. If the peptide being measured is not synthesized *in vivo* (as a result of a change in the steady state expression of the latent EBV genome) then one would not see a change in antibody titers to that protein (2).

Analysis of a subsample of participants in an ongoing study of puberty and psychopathology reveals that, among the sample of seropositive girls, traumatic life events are significantly related to increased levels of EBV p18-VCA antibodies, suggesting lower levels of cell-mediated immune function. Girls in situations of high life strain stand out as the group that manifests immunosuppression associated with life events.

EBV ANTIBODIES IN WHOLE BLOOD SPOTS

The significant effect of traumatic life events is consistent with previous findings linking life events to increased EBV antibody titers in adults (28, 37). In children, adverse life events have previously been associated with reduced NK cell activity (19), whereas parental separation or divorce has been correlated with reduced bacterial killing (21). The association between life events and immune function under conditions of high life strain may be analogous to recent work reporting that background life stress increases one's sensitivity to the immunomodulating effects of acute stressors (38, 39). Alternatively, life strain and traumatic life events in this study may represent two interdependent measures of stressful life circumstances, with additive effects on p18-VCA antibody level.

The sex-specific pattern of results is consistent with previous work in this population that demonstrated the development of a sex-differentiated relationship between life stress and symptoms of depression (25). Few psychoneuroimmunology studies report sex differences in stress-induced immunosuppression, although marital discord and negative interactions have recently been shown to have greater physiological impact on women than men (5, 40). Our findings may indicate a similar sex difference in sensitivity to social stress in late childhood and adolescence.

To date, few studies have considered the relationships between stress and immune function in children and adolescents, and fewer still have addressed these issues in large, community-based samples. We suggest that attention to gender, developmental status, and social context may enrich future psychoneuroimmunological studies, whereas blood spot methods can facilitate the collection of samples in future epidemiological studies that complement clinical and experimental designs.

This work was supported by Duke University Leon Lowenstein Center (A.A.); National Institute of Mental Health Grant RO1 MH48085 (E.J.C.); John D. and Catherine T. MacArthur Foundation (J.T.C.); and W.T. Grant Foundation Grant 92-1489-92 and 94-1489-94 (C.M.W.).

REFERENCES

1. Glaser R, Kiecolt-Glaser JK, Speicher CE, Holliday JE. Stress, loneliness, and changes in herpesvirus latency. *J Behav Med* 1985;8:249–60.
2. Glaser R, Pearson GR, Jones JF, Hillhouse J, Kennedy S, Mao H, Kiecolt-Glaser JK. Stress-related activation of Epstein-Barr virus. *Brain Behav Immun* 1991;5:219–32.
3. Glaser R, Pearson GR, Bonneau RH, Esterling BA, Atkinson C, Kiecolt-Glaser JK. Stress and the memory T-cell response to the Epstein-Barr virus in healthy medical students. *Health Psychol* 1993;12:435–42.
4. Kiecolt-Glaser JK, Glaser R, Shuttleworth EC, Dyer CS, Ogrocki P, Speicher CE. Chronic stress and immunity in family caregivers of Alzheimer's disease victims. *Psychosom Med* 1987;49:523–35.
5. Kiecolt-Glaser JK, Malarkey WB, Cacioppo JT, Glaser R. Stressful personal relationships: immune and endocrine function. In: Glaser R, Kiecolt-Glaser JK, editors. *Handbook of human stress and immunity*. San Diego (CA): Academic Press; 1994. p. 321–39.
6. Esterling BA, Antoni MH, Schneiderman N, Carver CS, LaPerriere A, Ironson G, Klimas NG, Fletcher MA. Psychosocial modulation of antibody to Epstein-Barr viral capsid antigen and human Herpesvirus Type-6 in HIV-1-infected and at-risk gay men. *Psychosom Med* 1992;54:354–71.
7. Dolken G, Weitzmann U, Boldt C, Bitzer M, Brugger W, Lohr GW. Enzyme-linked immunosorbent assay for IgG antibodies to Epstein-Barr virus-associated early antigens and viral capsid antigen. *J Immunol Methods* 1984;67:225–33.
8. Stevens R, Pass K, Fuller S, Wiznia A, Noble L, SD, Neal M. Blood spot screening and confirmatory tests for syphilis antibody. *J Clin Microbiol* 1992;30:2353–8.
9. Farzadegan H, Quinn T, Polk BF. Detecting antibodies to human immunodeficiency virus in dried blood on filter papers. *J Infect Dis* 1987;155:1073–4.
10. Worthman CM, Stallings JF. Measurement of gonadotropins in dried blood spots. *Clin Chem* 1994;40:448–53.
11. Worthman CM, Stallings JF. Hormone measures in finger-prick blood spot samples: new field methods for reproductive endocrinology. *Am J Phys Anthropol* 1997;104:1–22.
12. Varnier OE, Lillo FB, Reina S, DeMaria A, Terragna A, Schito G. Whole blood collection on filter paper is an effective means of obtaining samples for human immunodeficiency virus antibody assay. *AIDS Res Hum Retroviruses* 1988;4:131–6.
13. Thanasekaraan V, Wiseman MS, Rayner RJ, Hiller EJ, Shale DJ. Pseudomonas aeruginosa antibodies in blood spots from patients with cystic fibrosis. *Arch Dis Child* 1989;64:1599–603.
14. Jones JF, Straus SE. Chronic Epstein-Barr virus infection. *Annu Rev Med* 1987;38:195–209.
15. Glaser R, Pearl DK, Kiecolt-Glaser JK, Malarkey WB. Plasma cortisol levels and reactivation of latent Epstein-Barr virus in response to examination stress. *Psychoneuroendocrinology* 1994;19:765–72.
16. Glaser R, Rice J, Sheridan J, Fertel R, Stout J, Speicher CE, Pinsky D, Kotur M, Post A, Beck M, Kiecolt-Glaser JK. Stress-related immune suppression: health implications. *Brain Behav Immun* 1987;1:7–20.
17. Schleifer SJ, Scott B, Stein M, Keller SE. Behavioral and developmental aspects of immunity. *J Am Acad Child Psychiatry* 1986;26:751–63.
18. Targum SD, Clarkson LL, Magac-Harris K, Marshall LE, Skwerer RG. Measurement of cortisol and lymphocyte subpopulations in depressed and conduct-disordered adolescents. *J Affect Disord* 1990;18:91–6.
19. Birmaher B, Rabin BS, Garcia MR, Jain U, Whiteside TL, Williamson DE, Al-Shabbout M, Nelson BC, Dahl RE, Ryan ND. Cellular immunity in depressed, conduct disorder, and normal adolescents: role of adverse life events. *J Am Acad Child Adolesc Psychiatry* 1994;33:671–8.
20. Bartlett JA, Schleifer SJ, Demetrikopoulos MK, Keller SE. Immune differences in children with and without depression. *Biol Psychiatry* 1995;38:771–4.
21. Bartlett JA, Demetrikopoulos MK, Schleifer SJ, Keller SE. Phagocytosis and killing of *Staphylococcus aureus*: effects of stress

- and depression in children. *Clin Diagn Lab Immunol* 1997;4:362–6.
22. Boyce WT, Chesterman EA, Martin N, Folkman S, Cohen F, Wara D. Immunologic changes occurring at kindergarten entry predict respiratory illnesses after the Loma Prieta earthquake. *J Dev Behav Pediatr* 1993;14:296–303.
 23. Boyce WT, Chesney M, Alkon A, Tschann JM, Adams S, Chesterman B, Cohen F, Kaiser P, Folkman S, Wara D. Psychobiologic reactivity to stress and childhood respiratory illnesses: results of two prospective studies. *Psychosom Med* 1995;57:411–22.
 24. Costello EJ, Angold A, Burns B, Stangl DK, Tweed DL, Erkanli A, Worthman CM. The Great Smoky Mountains Study of Youth: goals, design, methods, and the prevalence of DSM-III-R disorders. *Arch Gen Psychiatry* 1996;53:1129–36.
 25. Angold A, Costello EJ, Worthman, CM. Puberty and depression: the roles of age, pubertal status and pubertal timing. *Psychol Med* 1998;28:51–61.
 26. Angold A, Prendergast M, Cox A, Harrington R, Simonoff E, Rutter M. The Child and Adolescent Psychiatric Assessment (CAPA). *Psychol Med* 1995;25:739–53.
 27. Henle W, Henle G. Epstein-Barr virus and infectious mononucleosis. In: Glaser R, Gottlieb-Stematsky T, editors. *Human herpesvirus infections: clinical aspects*. New York: Marcel Dekker; 1982. p. 151–62.
 28. Herbert TB, Cohen S. Stress and immunity in humans: a meta-analytic review. *Psychosom Med* 1993;55:364–79.
 29. Kiecolt-Glaser JK, Glaser R, Shuttlesworth EC, Dyer GS, Ogrocki P, Speicher CE. Chronic stress and immunity in family caregivers of Alzheimer's disease victims. *Psychosom Med* 1987;49:523–35.
 30. Kiecolt-Glaser JK, Fisher LD, Ogrocki P, Stout JC, Speicher CE, Glaser R. Marital quality, marital disruption, and immune function. *Psychosom Med* 1987;49:13–34.
 31. Kiecolt-Glaser JK, Kennedy S, Malkoff S, Fisher L, Speicher CE, Glaser R. Marital discord and immunity in males. *Psychosom Med* 1988;50:213–29.
 32. Esterling BA, Antoni MH, Kumar M, Schneiderman N. Defensiveness, trait anxiety, and Epstein-Barr viral capsid antigen antibody titers in healthy college students. *Health Psychol* 1993;12:132–9.
 33. Payne, DA, Mehta, SK, Tyring, SK, Stowe, RP, Pierson, D. Incidence of Epstein-Barr virus in astronaut saliva during space flight. *Aviat Space Environ Med* 1999;70:1211–3.
 34. Lutgendorf SK, Antoni MH, Kumar M, Schneiderman N. Changes in cognitive coping strategies predict EBV-antibody titer change following a stressor disclosure induction. *J Psychosom Med* 1994;38:63–78.
 35. Worthman CM, Stallings JF, Gubernick D. Measurement of hormones in blood spots: a nonisotopic assay for prolactin. *Am J Phys Anthropol* 1991;84:186–7.
 36. McDade TW. Culture change, stress, and immune function in Western Samoan youth [dissertation]. Atlanta (GA): Emory University; 1999.
 37. Kiecolt-Glaser JK, Glaser R. Stress and immune function in humans. In: Ader R, Felten DL, Cohen N, editors. *Psychoneuro-immunology*. New York: Academic Press; 1991. p. 849–67.
 38. Brosschaft JF, Benschop RJ, Godaert GLR, Olff M, DeSmet M, Hiejnen CJ, Ballieux RE. Influence of life stress on immunological reactivity to mild psychological stress. *Psychosom Med* 1994;56:216–24.
 39. Pike JL, Smith TL, Hauger RL, Nicassio PM, Patterson TL, McClintick J, Costlow C, Irwin MR. Chronic life stress alters sympathetic, neuroendocrine, and immune responsiveness to an acute psychological stressor in humans. *Psychosom Med* 1997;59:447–57.
 40. Kiecolt-Glaser JK, Malarkey WB, Chee MA, Newton T, Cacioppo JT, Mao HY, Glaser R. Negative behavior during marital conflict is associated with immunological down-regulation. *Psychosom Med* 1993;55:395–409.