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Measuring immune function:

Markers of cell-mediated immunity and inflammation in dried blood spots

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## **Introduction**

Stress is an important determinant of human immune function, but population-level research on the social ecology of immunity has lagged behind other areas of investigation. In large part this is due to methodological constraints associated with the assessment of immune function: venipuncture is a relatively invasive blood sampling procedure that requires the skills of a trained medical professional, and once collected, blood samples must be immediately assayed, or centrifuged, separated, and frozen. Obviously, these are serious impediments to field-based research, and they have hindered the exploration of stress-immunity relationships in large, representative, community-based studies.

Dried blood spots—samples of whole blood collected on filter paper following a simple finger prick—provide an alternative, minimally-invasive sampling procedure. Several community-based applications have shown this to be a convenient and reliable means to facilitate sample collection, storage, and transportation, and laboratory methods have been validated for a growing number of analytes (Worthman and Stallings 1994; Worthman and Stallings 1997; Cook et al. 1998; McDade et al. 2000; Erhardt et al. 2002; McDade and Shell-Duncan 2002; McDade et al. 2004). Also referred to as “Guthrie papers,” filter papers have been a core component of U.S. hospital-based newborn screening programs since the 1960s, with all newborns providing a blood spot sample to screen for congenital metabolic disorders (Mei et al. 2001).

In this chapter, detailed information is provided on the collection, handling, and analysis of dried blood spot samples for two measures of immune activity: Epstein-Barr virus antibodies and C-reactive protein. Minimally-invasive methods facilitate research with diverse populations outside the confines of the clinic or the lab, and results from the first field application of these methods are shared to demonstrate their utility for future field-based research.

### **Stress, immune function, and disease**

There is compelling evidence that stress-induced alterations in immune function are causally related to a range of disease outcomes (Kiecolt-Glaser and Glaser 1995; Cohen et al. 1998; Moynihan et al. 1998; Biondi 2001). The majority of research on the physiological pathways linking stress, immune function, and morbidity is conducted with animal models, but epidemiological as well as experimental evidence suggests analogous processes are at work in humans. Infection has been the most intensively investigated disease outcome, with stress and immunity linked to the common cold, tuberculosis, genital herpes, infectious mononucleosis, and HIV progression. Psychosocial stress has also been associated with reduced response to vaccination, a model that mimics the real-world process of pathogen exposure and immune response that is critical in defining resistance to infectious disease (Glaser et al. 1992; Petrie et al. 1995). Pathogen exposure alone is not sufficient to predict the occurrence or severity of infection, and psychosocial stress may in many cases be a critical cofactor (Meyer and Haggerty 1962; Boyce et al. 1977; Cohen 1995).

Immune processes are involved in response to injury, and a series of experiments has demonstrated that the rate of wound healing is slowed by psychosocial stress. For example, in students given a standardized punch biopsy wound, healing took on average 3 days longer when the wound was administered prior to a major exam than it did over the summer (Marucha et al. 1998). For older individuals caring for a relative with Alzheimer’s disease, wound healing was delayed almost 10 days compared to age-matched controls (Kiecolt-Glaser et al. 1995).

Causal linkages among stress, immunity, and risk for cancer in humans are more controversial. Animal models indicate that stress-induced immunosuppression can promote metastasis and decrease survival. Results with humans are less conclusive, but suggest that stressors such as bereavement or lack of social support may contribute to disease initiation or progression (Biondi et al. 1996; Biondi 2001). Similarly, evidence is mixed for autoimmune diseases, although a number of studies have linked psychosocial factors to alterations in immunity and disease symptomatology in rheumatoid arthritis (Affleck et al. 1997; Zautra et al. 1997).

The association between psychosocial stress and cardiovascular disease (CVD) has been established for some time, but immune processes have only recently been implicated as potential causal mechanisms. Current research has indicated that CVD may have a substantial inflammatory component, with immunologically-mediated processes centrally involved in disease initiation and progression (Tracy 1998; Ross 1999; Libby et al. 2002). In particular, cytokines such as IL-1 $\beta$ , IL-6, and TNF $\alpha$  may contribute to the development of atherosclerotic lesions, and recent research has suggested that psychosocial stressors can upregulate production of pro-inflammatory cytokines (Black 2002). This is a new area of research, and the potential contribution of stress-induced modulation of inflammatory processes to risk for CVD and other chronic diseases remains to be thoroughly investigated.

In sum, experimental as well as observational research highlights the relevance of stress and immune function to a range of disease processes. However, negative findings are also present, and in many cases logistical and ethical considerations have limited attempts to establish direct links between stress, immunity, and disease in humans.

### **Current research in psychoneuroimmunology**

Psychoneuroimmunologists have employed a number of measures of immunity to investigate the effects of psychosocial stress (Schleifer et al. 1986; Herbert and Cohen 1993; Kiecolt-Glaser and Glaser 1997). Enumerative measures include the quantification of the number and/or percentage of various white blood cells in peripheral blood (helper T cells, cytotoxic/suppressor T cells, B cells, natural killer cells, monocytes), or the quantification of immunoglobulins in blood (primarily IgG, IgM, IgA) or saliva (secretory IgA). An additional method involves the measurement of specific antibodies, such as antibodies against resident herpes viruses (Glaser et al. 1985), or antibody responses following a vaccine challenge (Glaser et al. 1992; Petrie et al. 1995).

Commonly used functional assays of immune competence include lymphocyte proliferation and natural killer (NK) cell cytotoxic activity. To measure proliferative responsiveness (also referred to as blastogenesis), lymphocytes are incubated with mitogens that induce non-specific T and B cell division and replication. Reduced proliferation reflects down-regulated immune activity that has been associated with a range of immunodeficiency conditions (Kiecolt-Glaser and Glaser 1997). Recently, there has been increased interest in investigating patterns of cytokine and cytokine receptor production following lymphocyte activation as additional functional measures of immunity (Marshall and al. 1998; Glaser and al. 2001). NK cell activity is evaluated by growing target cells (typically from a tumor cell line) in media, and then measuring the ability of NK cells to lyse the target cells. NK cell activity is thought to be important for protecting the body against damaged or altered (i.e., infected or cancerous) cells.

For the most part, these protocols require the collection of large volumes of blood through venipuncture, and prompt access to laboratory facilities for the processing and analysis

of samples. This has served as a major constraint on attempts to conduct field-based, population-level research on stress and immunity, and is a primary reason why the vast majority of current work is based in laboratory or clinical settings, with relatively homogenous, opportunistic samples. A recent literature review underscores this point, stating “The typical experimental subject in psychoimmunology is a young, male, Caucasian, healthy, medical or psychology student, probably a light or nonsmoker, consuming little or no alcohol or coffee, with no history of allergy or recent infectious disease. . .” (p. 202 Biondi 2001).

Despite the methodological obstacles, a number of investigators have attempted to consider a range of stressors occurring in more naturalistic circumstances. While these studies cannot attain the high degree of control possible in the laboratory, they strive for increased generalizability and external validity. Some of the earliest PNI work investigated changes in immune function surrounding bereavement, and found consistent impairments in immune function following the loss of a loved one (Irwin et al. 1987). An early series of studies investigating changes in immunity during medical school examinations has also been important, with exam stress associated with impairments in multiple measures of immune function (Glaser et al. 1985; Jemmott and Magloire 1988; Glaser et al. 1994). These studies also show moderating effects of social support. More recent studies have considered the immunological impact of the stress and life disruption associated with natural disasters (earthquake; hurricane) (Boyce et al. 1993; Ironson et al. 1997; Solomon et al. 1997).

An additional series of studies explores the immunosuppressive effects of stressful personal relationships (Kiecolt-Glaser et al. 1994). Recent divorce and self-reports of poor marriage quality have been related to increased levels of herpesvirus antibodies (indicative of suppressed cell-mediated immunity), and spousal caregivers of Alzheimer's disease patients report more days of illness, have higher herpesvirus levels, heal more slowly following an experimentally-induced wound, and are less likely to produce antibodies in response to a influenza vaccine than age-matched controls (Kiecolt-Glaser et al. 1995; Kiecolt-Glaser et al. 1996). Caregivers over the age of 70 show significantly greater reductions in immune function than younger caregivers.

The evidence linking stress to human immune function is relatively strong and consistent. A meta-analysis of this literature (Herbert and Cohen 1993) shows that stress is significantly associated with decreased numbers of T, B, and NK cells, suppressed lymphocyte proliferation and cytotoxic activity, and lower levels of secretory IgA and IgM. Antibodies to resident herpesviruses are elevated under stress, consistent with the interpretation that stress suppresses cell-mediated immune function. Based on this meta-analysis, cautious conclusions can be drawn regarding the impact of different types of stressors: 1) objective events appear to have greater effects than self-reported events, 2) long-term stressors have more consistent negative effects than acute stressors, indicating the absence of adaptation immunologically to stress, and 3) social and non-social stressors have different immunological consequences.

In addition, although this has not been a major focus of research, constitutional and/or contextual factors may influence an individual's sensitivity to stress-induced immune modulation. For example, age, personality factors, presence of an immunosuppressive disease such as AIDS, physiological reactivity to an acute stressor, levels of chronic background stress, and social support may moderate the effects of stress on immune function and disease (Boyce et al. 1995; Kiecolt-Glaser and Glaser 1995; Kiecolt-Glaser et al. 1996; Uchino et al. 1996; Pike et al. 1997; Segerstrom et al. 2001).

Prior research in PNI has demonstrated that psychosocial processes are causally related to important aspects of human immune function. The assessment of immunity is complicated by the fact that the immune system is comprised of multiple, inter-related subsystems of defense, and therefore no single measure can provide a global assessment of immunocompetence. Add to this the relative invasiveness of current methods of immunological assessment, and it is clear why the majority of research on stress and immune function has been limited to clinical or laboratory settings.

### **Measuring cell-mediated immunity:**

#### **Antibodies against the Epstein-Barr virus in dried blood spots**

A practical method for assessing immunity in field-based stress research must overcome the logistical constraints associated with venipuncture without sacrificing validity or reliability. Whole blood spots collected from a simple finger prick provide a minimally-invasive alternative for collecting blood, and antibodies against the Epstein-Barr virus (EBV) can be quantified as an indirect measure of cell-mediated immunity.

#### **The EBV antibody model**

The Epstein-Barr virus is a ubiquitous herpesvirus to which approximately 90% of adults in industrialized nations, and nearly 100% of adults in developing nations, have been exposed (Henle and Henle 1982). Once infected, individuals permanently harbor EBV, and adequate cell-mediated immune function is critical for maintaining the virus in a latent state. Stress-induced immunosuppression allows EBV to reactivate and release viral antigens into circulation, to which a humoral antibody response may emerge (Glaser et al. 1991). As a result, levels of antibodies against EBV antigens provide an indirect measure of an aspect of cell-mediated immune function, such that increased EBV antibody titers indicate lower cell-mediated immunity (Figure 1).

Although this model may at first seem counterintuitive—an *increase* in EBV antibody titer, itself an aspect of immune function, is interpreted as indicating a *decrease* in cell-mediated immune performance—it is important to recognize that cell-mediated immune processes are primarily involved in maintaining viral latency, and that humoral-mediated immunity (including the production of antibodies) represents a second line of defense that is called upon when cell-mediated processes fail to control the virus. In addition, increased EBV antibody titers have been associated with reduced EBV-specific T-cell proliferation and reduced cytotoxic T cell lysis of EBV-infected target cells, consistent with the interpretation that EBV antibodies reflect broader cell-mediated immune performance (Glaser et al. 1987; Glaser et al. 1993).

The utility of the EBV antibody model has been demonstrated in a number of studies of stress-induced immunosuppression (Table 1). Increased antibody titers have been associated with a wide range of stressors, including medical school exams (Glaser et al. 1987; Glaser et al. 1993), involvement in a poor quality marriage (Kiecolt-Glaser et al. 1987; Kiecolt-Glaser et al. 1988), and caring for a family member with Alzheimer's disease (Kiecolt-Glaser et al. 1987). Additionally, loneliness, defensiveness, and anxiety have all been positively associated with EBV antibody titers (Glaser et al. 1985; Esterling et al. 1993). Conversely, stress management interventions and disclosure of previously repressed trauma have been associated with decreases in EBV antibodies (Esterling et al. 1992; Lutgendorf et al. 1994). Furthermore, in comparison with other measures of immunity, meta-analysis has identified EBV antibodies as among the strongest and most consistent correlates of chronic stress (Herbert and Cohen 1993). Although

relatively few studies in psychoneuroimmunology make explicit links to disease outcomes (Keller et al. 1994), relevant outcomes for the EBV antibody model include symptoms of infectious disease, neoplastic disease, and rates of wound healing.

Like memory responses to other previously encountered antigens, EBV-specific antibody titers can be expected to rise 2-7 days after viral antigen re-exposure (Kuby 1994). As such, the duration of time elapsing between stressor and EBV antibody response is on the order of days or weeks. EBV antibody levels are therefore not subject to short-term fluctuation, acute context effects, or diurnal variation, and a single sample can be used as an immunological measure of chronic stress. This is an advantage over other stress measures which require multiple samples, or are sensitive to the circumstances under which they are collected.

Any attempt to investigate the direct effects of psychosocial stress on immune function must pay careful attention to potential confounders such as nutritional status and current infection, both of which can independently modulate immunity (Kiecolt-Glaser and Glaser 1988). The majority of research in PNI sidesteps this issue by recruiting only healthy individuals, but this may not always be the case for field-based research that includes larger, more representative samples from a wider range of populations. Recent research has also suggested that sleep quality may be a potential mediator of stress-immune relationships (Hall et al. 1998).

### **EBV antibodies in dried blood spots**

#### *Sample collection, transport, and storage*

The requirements for collecting and processing blood spot samples are relatively minimal (Add Figure 2 if B&W photos are possible). First, the participant's finger is cleaned with isopropyl alcohol, and then pricked with a sterile, disposable lancet that is commonly used by diabetics to monitor blood glucose (Microtainer, Franklin Lakes, NJ). The lancet is designed to deliver a controlled, uniform puncture that will stimulate sufficient capillary blood flow with minimal injury. The first drop of blood is wiped away, and up to five drops (~50uL per drop) are spotted onto standardized filter paper (Schleicher and Schuell #903, Keene, NH). The filter paper is certified to meet performance standards for sample absorption and lot-to-lot consistency set by the National Committee on Clinical Laboratory Standards, and by the Food and Drug Administration regulations for Class II Medical Devices. The samples are allowed to dry (four hours to overnight), at which point they can be stacked and stored in sealed plastic bags or plastic containers with desiccant.

This is a relatively painless, non-invasive, and convenient protocol for collecting and processing blood samples, particularly in comparison with venipuncture. Samples do not need to be centrifuged, separated, or immediately frozen, and samples can be stored and transported in air-tight containers at ambient temperatures (although elevated temperatures should be avoided). In addition, finger prick blood sampling can be performed by non-medically trained personnel, and samples can be collected virtually anywhere. Infants, children, and the elderly can provide blood without great difficulty, and repeat sampling becomes more feasible.

Requirements for shipping blood spot samples are relatively minimal unless the samples are known to contain an infectious or etiologic agent. Samples from normal, healthy individuals are considered "diagnostic specimens," and according to current regulations, can be shipped without special packaging, labeling, or permitting. With respect to importing samples from overseas, the Centers for Disease Control and Prevention (CDC) will issue importation permits, although such permits may only be required under certain circumstances. Up-to-date shipping

and importation guidelines are available from the CDC (<http://www.cdc.gov/od/ohs/biosfty/biosfty.htm>).

Prior work has shown that investigators can attain the same degree of precision and reliability with blood spots that they achieve with standard methods of sample collection such as venipuncture (Mei et al. 2001). However, important sources of potential error should be acknowledged. First, proper placement of whole blood on the filter paper is essential. The uniform absorbing properties of the filter paper will be defeated if blood is blotted or smeared onto the paper, or if a drop of blood is placed on top of a previously collected drop. In addition, the volume of whole blood applied to filter paper as a blood spot can influence the volume of serum contained within a single disc punched out of that spot (Adam et al. 2000). For this reason, an attempt should be made to ensure that all blood spots used for analyses are of a comparable size. Variation in blood spot size can be minimized by collecting samples on filter papers with pre-printed circles as guides (available from Schleicher & Schuell) to standardize the volume of whole blood collected from each individual. When filled to its border, each circle will contain approximately 50 $\mu$ L of whole blood.

The filter paper matrix stabilizes most analytes in dried blood spots, but the rate of sample degradation will vary by analyte and should be evaluated prior to sample collection. To determine the stability of EBV antibodies in dried blood spots, a set of samples was exposed to one of three temperature conditions (4°C, room temperature, 37°C) for varying lengths of time up to eight weeks (McDade et al. 2000). Matched “baseline” samples served as the basis for comparison, and were frozen at –23°C immediately after collection and drying. EBV antibodies were found to be stable in dried blood spots for at least 8 weeks if stored at room temperature or at 4°C. However, samples began to deteriorate after 1 week of storage at 37°C. Blood spot samples collected for analysis of EBV antibodies should therefore be protected from excessive heat exposure. While it is always advisable to refrigerate or freeze samples when possible to minimize the chances of degradation, the stability of EBV antibodies in dried blood spots at room temperature provides flexibility in the collection of samples in field settings. The impact of repeated freezing and thawing on sample stability was also evaluated, with no evidence of deterioration in EBV antibody concentrations after six cycles of freeze/thaw.

### *Laboratory analysis*

The dried blood spot EBV antibody protocol is an adaptation of a commercially available enzyme-linked immunosorbent assay (ELISA) for measuring IgG antibodies against the EBV viral capsid antigen (VCA) complex (McDade et al. 2000). Beyond the materials provided with the assay kit, equipment requirements include access to a microplate spectrophotometer and a microplate washer, as well as equipment standard to most wet labs: deionized water, single channel and repeater pipettors, incubator, and facilities for biohazardous waste disposal.

The day before an assay is to be performed, blood spot samples are removed from the freezer, and a small hole punch (available from office supply stores) is used to punch out one 3.2 mm disc of whole blood. The disk is transferred to a small glass test tube and incubated overnight in buffer, during which time the dried whole blood elutes from the disc and into solution. This reconstituted sample is then added in duplicate to microtiter wells. Antigen-antibody complexes form between EBV-VCA IgG antibodies present in the sample and synthetic peptide p18 bound to the surface of the wells. Horseradish peroxidase-labeled anti-human IgG in the presence of a chromogen substrate reacts with the antigen-antibody complex resulting in color development. The concentration of EBV-VCA IgG antibodies is directly related to the

absorbance of the solution measured at 450nm. Sample values are interpolated from a standard curve using a linear data reduction protocol, and reported in ELISA units.

The accurate determination of previous exposure to EBV, and subsequent seroconversion, is critical since the model linking stress to suppressed cell-mediated immune function and increased EBV antibody level does not apply to seronegative individuals. Analyses must therefore focus exclusively on seropositive individuals. Previous comparison of matched plasma and blood spot samples for seronegative and seropositive individuals established a blood spot seropositivity cut-off value of 20 ELISA units (McDade et al. 2000). Individuals with EBV antibody levels below this value are assumed to be seronegative for EBV, and must be excluded from analysis.

Previous analysis of EBV antibody concentration in a set of matched plasma and blood spot samples indicates a high level of agreement across these two methods (Pearson  $R=0.97$ ,  $N=40$ ) (McDade et al. 2000). In addition, the blood spot method has good precision and reliability, demonstrating within-assay and between-assay percent coefficients of variation of less than 10 percent. The assay for quantifying EBV antibodies in dried blood spots has been well-validated, and represents a viable alternative to methods requiring serum or plasma.

## **Measuring inflammation: CRP in dried blood spots**

### *The CRP model*

C-reactive protein is an inflammatory protein produced by liver hepatocytes in response to messenger cytokines, primarily IL-6 (Ballou and Kushner 1992; Pepys 1995). CRP is an important component of innate immunity, and has been used clinically for decades as an indicator of active infection. Recent development of high sensitivity CRP assays has led to the discovery that slight elevations of CRP—in the range of what was previously considered normal—are indicative of low-grade inflammatory processes that may be related to the pathophysiology of cardiovascular disease.

More than a dozen population-based studies have demonstrated that elevated CRP at baseline is a significant predictor of future cardiovascular disease, even after adjusting for other standard risk factors (Ridker et al. 1998; Lagrand et al. 1999; Libby et al. 2002; Danesh et al. 2004). While prior practice often employed 10 mg/L as a reference value for identifying active inflammatory conditions, these recent studies suggest that cardiovascular risk may increase significantly with baseline concentrations of CRP greater than 1 to 2 mg/L (Pearson et al. 2003).

Research on the impact of psychosocial stress on inflammatory pathways is in its early stages, with evidence thus far indicating that this may be an important area for future work (Black 2002). A number of studies have reported significant associations between symptoms of depression and markers of inflammatory activity, including IL-6, TNF $\alpha$ , and CRP (Dentino et al. 1999; Danner et al. 2003; Glaser et al. 2003). Measures of burnout, self-reported distress, and post-traumatic stress disorder have also been linked with inflammation (Lutgendorf et al. 1999; Maes et al. 1999; Grossi et al. 2003), as has the chronic stress associated with caring for a relative with dementia (Kiecolt-Glaser et al. 2003). Increased concentrations of CRP have also been reported in individuals of low socio-economic status, independent of a number of potentially confounding health behaviors (Owen et al. 2003). Downstream health outcomes relevant to research on stress and inflammation include symptoms of cardiovascular and metabolic disease.

A recent review of indicators of inflammation conducted by the American Heart Association (AHA) and the Centers for Disease Control and Prevention (CDC) has

recommended the limited measurement of CRP in clinical practice, and called for additional population-based research (Pearson et al. 2003). They also propose cutpoints of low risk (<1.0 mg/L), average risk (1.0 to 3.0 mg/L), and high risk (>3.0 mg/L) that approximate the tertile distribution of serum/plasma CRP in a range of populations. Given the relative stability of CRP concentrations in individuals across time (Danesh et al. 2004), a single measure can provide meaningful information on the level of chronic inflammation. Serum/plasma concentrations of CRP greater than 10 mg/L are assumed to represent an acute inflammatory response, likely to an infectious agent. The AHA/CDC guidelines therefore recommend discarding these values, and collecting another sample two weeks later that will provide a better indicator of chronic inflammation (Pearson et al. 2003).

This raises a number of issues that should be considered prior to the application of blood spot CRP to field-based research on stress and inflammation. First, repeat sampling is probably not feasible in population research given that it is highly unlikely that samples will be analyzed within two weeks of collection. Therefore, if the AHA/CDC guidelines are followed, a number of individuals with high CRP will have to be removed from the sample, possibly introducing bias. Second, the AHA/CDC cut-off values are based on concentrations of CRP measured in serum or plasma, and these concentrations will differ significantly in blood spot samples. This can be addressed by using blood spot CRP cut-off values based on population-specific tertiles, or by converting blood spot results to plasma equivalents using previously established conversion factors (McDade et al. 2004). Lastly, smoking, obesity, and intense physical activity are associated with increased CRP, and may confound the association between stress and inflammation.

### **CRP in dried blood spots**

#### *Sample collection, transport, and storage*

The requirements for collecting and processing blood spot samples for CRP analysis are identical to those for EBV antibodies. The only exception is that CRP degrades more quickly, and therefore requires a higher degree of protection from heat exposure than samples used only for analysis of EBV antibodies. In an evaluation of sample integrity over a 14 day period, it was determined that CRP remains stable for at least 14 days at room temperature or at 4°C, but deteriorates significantly after 3 days at 37°C, or 3 days in an oscillating condition of 12 hours at 32°C and 12 hours at 22°C (simulating diurnal temperature variation in tropical environments) (McDade et al. 2004). As with EBV antibodies, blood spot CRP is robust to cycles of freeze/thaw, with no deterioration detected after five cycles.

Seven 3.2 mm discs can be punched out of a single full size drop of whole blood (~50uL) collected on filter paper. Since the CRP and EBV antibody assays each require only one disc, both assays can be performed from a single drop, with sample to spare. However, it is advisable to collect more than one drop in case samples need to be re-analyzed, and to allow for measurement of additional analytes in the future.

#### *Laboratory analysis*

Blood spot CRP is assayed using a high sensitivity enzyme immunoassay protocol developed from commercially available reagents (McDade et al. 2004). Equipment requirements include a microplate reader and washer, a hematology centrifuge, pH meter, and standard wet lab resources.

In comparison with the EBV antibody assay, the blood spot CRP protocol requires a higher degree of technical proficiency in the lab, primarily due to the fact that blood spot standards are made by adding washed erythrocytes to a serum-based reference preparation with known concentration of CRP. Washed erythrocytes are obtained by collecting several milliliters of whole blood (EDTA-anticoagulated) from a single individual, centrifuging at 1,500 x g for 15 minutes, removing plasma and buffy coat, and rinsing the remaining erythrocytes in normal saline. This procedure is repeated three times, washed erythrocytes are added to the CRP reference preparation, mixed, and then spotted onto filter paper in 50 uL aliquots. Standards are then dried and stored at  $-30^{\circ}\text{C}$ . Adding washed erythrocytes to the CRP reference preparation maximizes comparability between assay standards and the whole blood matrix of blood spot samples. CRP standards can be prepared in batches in advance of sample analysis, and do not need to be made prior to each assay.

The day before an assay is to be performed, blood spot standards and samples are removed from the freezer, and one 3.2 mm disc of whole blood is eluted in buffer overnight at  $4^{\circ}\text{C}$ . Also the day before (or further in advance in batches, if desired), a microtiter plate is coated overnight with anti-human CRP antibodies. The day of the assay, blood spot eluate is pipetted in duplicate into microtiter wells. Through a series of incubation and wash steps, an antigen-antibody “sandwich” forms, with CRP from the sample located between peroxidase-conjugated anti-human CRP detection antibody and the capture antibody bound to the microtiter plate. Chromogen substrate reacts with this complex, resulting in color development proportional to the concentration of CRP. Absorbance is read at 490 nm, and CRP concentrations are calculated from the best fit 4-parameter logistic standard curve.

The performance characteristics of the blood spot CRP assay have been previously evaluated, and indicate that the assay has good sensitivity, precision, and reliability (McDade et al. 2004). The lower detection limit of the assay is 0.028 mg/L, well below the level needed to detect concentrations of CRP associated with cardiovascular disease risk. Assay performance was further evaluated by comparing CRP concentrations in 94 paired blood spot and serum samples. Serum samples were analyzed with a widely used clinical assay system (IMAGE<sup>TM</sup>, Beckman Coulter, Inc). The association between the blood spot and serum methods is linear and the correlation is high (Pearson  $R=0.96$ ).

### **Measuring immune function in the field**

The first field application of the blood spot EBV antibody method was conducted in rural North Carolina, as part of the ongoing Great Smoky Mountains Study of adolescent psychopathology and service use (McDade et al. 2000). Finger prick blood spot samples were already being collected as part of the in-home interview, and a subsample of 256 9-13 year-olds were selected to pilot the EBV antibody method. Of these individuals, 80.1% were seropositive for EBV. Fifty one individuals did not show evidence of prior EBV exposure (19.9%), and therefore had to be excluded from subsequent analyses. This is a significant limitation of the EBV antibody method that may introduce a degree of bias, and that limits the generalizability of results to the population of EBV-positive individuals. Fortunately, the likelihood of seropositivity increases with age, thereby reducing the number of individuals that must be excluded.

Following previous research in psychoneuroimmunology, we hypothesized that negative life events would be associated with reduced cell-mediated immunity, as indicated by higher EBV antibody titers. Negative life events included the death of a close family member or friend,

physical or sexual abuse, and exposure to a potentially traumatic experience (e.g., violence, accident). On average, participants had one negative life event in their lifetime (SD=1.2, range=0-6). Life events were not significantly associated with EBV antibodies in boys. However, for girls there was a strong positive association between negative life events and EBV antibodies (McDade et al. 2000). These analyses were the first to demonstrate an association between stress and EBV antibodies in a community-based setting.

Following these favorable results, the EBV antibody method was implemented in a non-western field setting for the first time in the islands of Samoa. Samoa was selected due to its high rates of adolescent suicide—suggesting an exceptionally high level of adolescent distress—and due to its low rates of malnutrition and endemic disease, two factors that may obscure stress-immune function relationships. The objectives of the study were threefold: 1) Evaluate the utility of the blood spot EBV antibody method for measuring stress and immunity in a remote setting; 2) Consider sources of stress for adolescents in Samoa and investigate their physiological impact; and 3) Introduce a cross-cultural, population-level perspective to current research in psychoneuroimmunology. Analyses from this dataset have led to a series of papers investigating the social and cultural ecology of adolescent stress in Samoa (McDade et al. 2000; McDade 2001; McDade 2002; McDade and Worthman 2004), but the discussion here is limited to life events, a dominant paradigm in current stress research.

It was hypothesized that negative life events would be associated with reduced immune function as previous research in western populations has demonstrated, but that the significance of these events would be defined by the local cultural context (McDade 2003). Participants were recruited from 14 villages across Samoa, and 295 individuals between the ages of 10 and 20 years provided data for the study, including a finger prick blood spot sample. Blood spots were collected in a centralized location in each village, dried overnight, stored in a refrigerator for up to two weeks, and then shipped via express mail to the US. Of the 295 participants, only 2 had to be removed due to lack of prior EBV exposure, reflecting a 99% seropositivity rate for this population.

Measures of anthropometric status indicated that individuals in the study were well-nourished, and no significant relationships were found with EBV antibody level (McDade et al. 2000). To verify this finding in the subsample of adolescents used in the analysis of life events, body mass index and skinfold measures were evaluated as predictors of immune function. Nutritional status was not significantly associated with EBV antibody concentration or the number of life events, indicating that it was not likely to confound the association between stress and immunity.

A less sensitive version of the blood spot CRP assay described above was used as a screening tool to identify individuals with a current or recent infection. CRP is a central component of the acute phase response, the body's first line of defense against infectious disease, and circulating concentrations increase by a factor of 100 to 1,000 during the 24 to 72 hours following an injury or infectious challenge (Fleck 1989; Baumann and Gauldie 1994). The half-life of circulating CRP is approximately 18 hours, and concentrations remain elevated during the course of infection for approximately one week following resolution (Gillespie et al. 1991; Mortensen 1994). Since CRP has been shown to increase in response to a wide range of pathogenic agents, it is a potentially useful marker of infectious burden. Of course, other methods may be applied to evaluate infection status, including physical exams and/or symptom diaries or recalls, although the CRP method has the advantage of being objective and less subject to recall bias.

Based on previous work in this population (McDade et al. 2000), a blood spot CRP concentration greater than or equal to 5 mg/L was used as a cut-off to identify individuals with a current or recent infection. Participants with elevated CRP (N=15) were removed from the sample prior to analysis to minimize the possibility of confounding. This is a conservative step that biases results toward the null, as individuals with current infection may be the same individuals who are suffering from the infectious consequences of stress-induced immunosuppression.

A summary life events variable was constructed based on the following events occurring in the preceding year: death of a family member or close friend; serious illness in the family; hospitalization of a family member; and the number of *faalavelave* hosted by the family (0=none, 1=one, 2=two or more). *Faalavelave* is a system of public, formalized gift exchange associated with community events such as weddings, funerals, and building dedications (Shore 1982; O'Meara 1990). Previous ethnographic work, as well as observations in the field based on a series of in-depth, semi-structured interviews, indicated that *faalavelave* can be a significant source of stress, particularly because it taxes a family's economic as well as social resources.

Life events were significantly related to EBV antibody titer in interaction with socioeconomic status (defined by parental occupation and remittances from relatives overseas). As expected, the association between life events and EBV antibody level was positive, such that adolescents with more life events had elevated antibody levels, indicating reduced cell-mediated immunity. This association was strongest for adolescents from families with low and middle levels of socioeconomic resources, whereas life events were not associated with suppressed immunity for adolescents with high resources. Figure 3 presents these results for *faalavelave*—the most frequently reported life event in the sample. For families with low economic resources, the costs of *faalavelave* may be particularly burdensome, forcing them to draw on the resources of extended kin networks in order to maintain their standing within the community. The economic and social debts that follow are likely to make these experiences more stressful for all members of the household.

These results are consistent with previous work in western populations linking life events to suppressed immune function (Herbert and Cohen 1993), and they demonstrate the feasibility and utility of measuring EBV antibodies and CRP in dried blood spots. These methods have since been applied to samples drawn from populations in Kenya, Bolivia, Russia, Kentucky, and Chicago.

### **Future Directions**

A constraint of blood spot methods is the relatively small volume of sample, which may limit the range of analytes that can be measured. For example, recent work suggests that patterns of cytokine expression may be important mediators of the associations among stress, immune function, and disease, but current assay protocols require volumes of blood (i.e., 50 or 100 uL of serum/plasma per cytokine) that are unattainable with blood spots. However, recent innovations in immunoassay technology allow the simultaneous quantification of multiple analytes in one sample, whereas previously only one analyte could be analyzed at a time. This advance is made possible by a multi-analyte flow analyzer, which uses different sets of polystyrene microspheres—pre-coated with a range of capture antibodies—to assay multiple analytes from a single sample.

Recent work has successfully used this method to measure up to 15 cytokines in less than a single drop of human plasma (Carson and Vignali 1999; Cook et al. 2001). The increased

sensitivity, reduced cost, and low sample volume requirements afforded by this technology promise to expand the range of factors that can be measured in whole blood spots. Application of this method is in its early stages, but its feasibility with blood spots has already been demonstrated (Bellisario et al. 2000). Much validation work remains, but this approach may facilitate the implementation of measures of cytokine activity into field-based research on stress and immunity.

### **Conclusion**

Dried blood spots provide a “field-friendly” option for collecting blood samples that minimizes participant inconvenience and burden, and eases requirements associated with sample collection, transportation, and storage. Two measures of immune activity—EBV antibodies and CRP—can be reliably measured in blood spots, with additional measures possible in the future, subject to assay development and validation. These methods make possible population-level research on stress and immunity that can complement current laboratory and clinic-based approaches.

Advantages of the blood spot EBV antibody and CRP methods include a minimally-invasive sampling protocol that can be implemented in even the most remote field settings. By bringing these methods to research participants, the impact of stress on immune function can be evaluated in larger, more diverse, and more representative samples. An additional advantage of the EBV antibody method is that a single measure has been shown to be among the strongest immunological correlates of chronic stress. As such, it provides insight into the effects of psychosocial processes on a critical physiological system, and it can be used to complement other physiological stress indicators (e.g., blood pressure, cortisol).

A disadvantage of all blood spot methods is that most research is conducted with serum or plasma samples, and comparisons of results across methods must be made with caution. However, the high correlation between blood spot and serum/plasma methods for assessing EBV antibodies and CRP makes this less of a concern. Another disadvantage is the fact that EBV antibodies provide only a single measure of cell-mediated immune activity, and CRP provides only a single measure of inflammation. A more complete picture of the impact of stress on immune function will require additional measures. Lastly, a limitation of the EBV antibody method is that it relies on prior exposure to EBV, and high rates of seronegativity will limit statistical power and may introduce bias. However, due to the ubiquity of EBV, this is only likely to be an problem in samples of younger children from affluent settings.

Research in North Carolina and Samoa has demonstrated the feasibility of population-level research on stress and immune function, and future work should consider developing new field measures, including specific cytokines, thymic peptides, and antibody responses to vaccine challenge. With an expanding range of methodological options, field-based research on the social ecology of immune function will yield important insights into the physiological and health implications of stress for individuals in the U.S. and overseas.

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Table 1. Previous research associating naturalistic stressors with EBV antibody titer.

Sample	Stressor	Association with EBV antibody	Reference
49 medical students 45 medical students	Major exams	↑	Glaser et al. 1985 Glaser et al. 1994
54 undergraduates	Self-reported anxiety or defensiveness	↑	Esterling et al. 1993
38 married women 32 married men	Poor quality marriage	↑	Kiecolt-Glaser et al. 1987 Kiecolt-Glaser et al. 1988
76 women 64 men	Recent separation/divorce	↑	Kiecolt-Glaser et al. 1987 Kiecolt-Glaser et al. 1988
90 newlywed couples 31 older married couples	Negative interactions during marital discussion	↑	Kiecolt-Glaser et al. 1993 Kiecolt-Glaser et al. 1997
29 West Point cadets	Basic training Final exams	↔ ↑	Glaser et al. 1999
68 adults	Caring for family member with Alzheimer's disease	↑	Kiecolt-Glaser et al. 1987
65 gay men	Stress management intervention	↓	Esterling et al. 1992
76 undergraduates	Disclosure of repressed trauma	↓	Lutgendorf et al. 1994

Figure 1. Model of the relationships among stress, cell-mediated immunity, and EBV antibody titer. An increase in EBV-specific antibodies indicates relative impairment of cell-mediated immune performance.

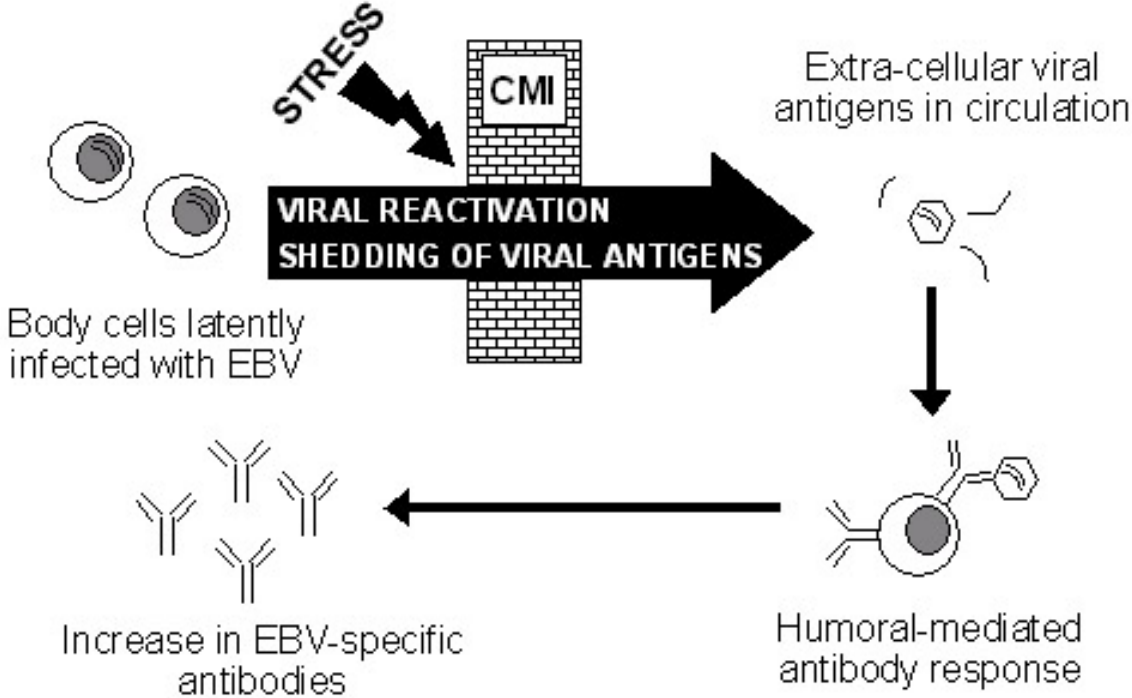
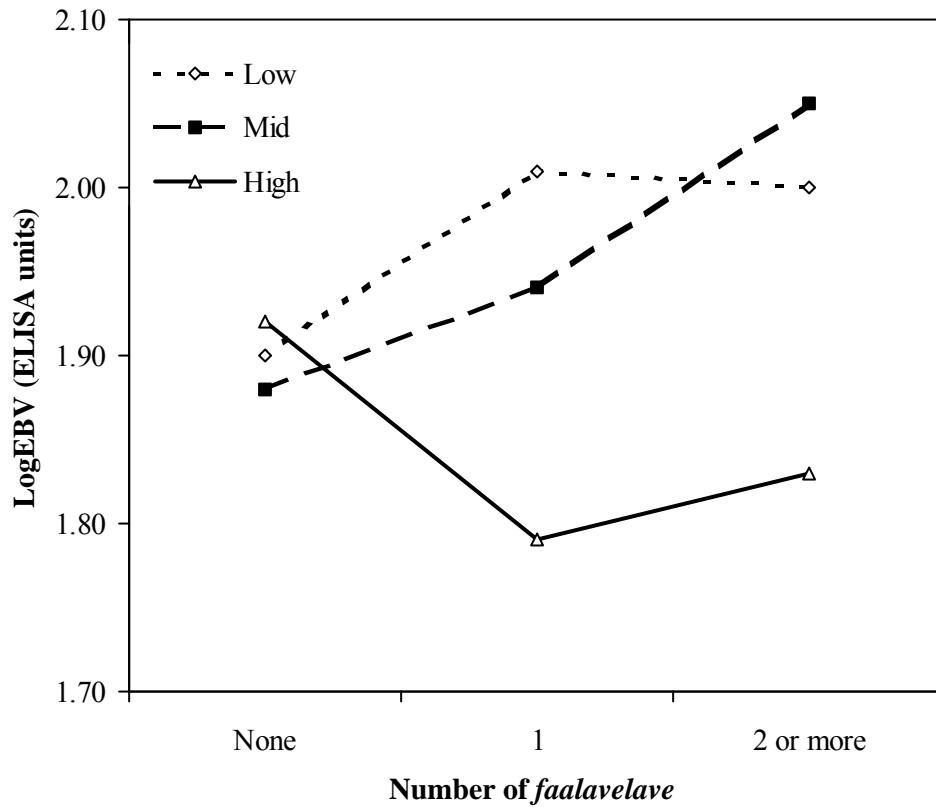


Figure 2. Procedure for collecting finger prick blood spot samples.



Figure 3. Interaction between number of *faalavelave* and socio-economic status in predicting EBV antibody titer in Samoan adolescents.



**Resource list**Sample collection

Lancets (#366357, Becton Dickinson, or equivalent)

Filter papers (#903, Schleicher & Schuell, Keene, NH)

EBV antibody analysis

ELISA kit (P001606A, DiaSorin, Stillwater, MN)

CRP analysis

Calibrator (#X0923, Dako, Carpinteria, CA)

Coating antibody (#A0073, Dako)

Detection antibody (#P227, Dako)

Microtiter plate (#439454, NUNC Maxisorp)

Chromogen substrate (#S2045, Dako)