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Autonomic and Glucocorticoid Associations with the Steady-State Expression of Latent Epstein–Barr Virus

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Previous studies have demonstrated the impact of psychological stress on the steady-state expression/reactivation of latent Epstein–Barr virus (EBV). Stress-induced decrements in the cellular immune response result in less control over the expression of the latent virus, resulting in increases in antibody to the virus. In Study 1, we investigated whether the steady-state expression of latent EBV *in vivo* differed between high and low stress reactors, as defined by sympathetic cardiac reactivity. Autonomic activity and antibody titers to Epstein–Barr virus capsid antigen (VCA) were measured in 50 elderly women latently infected with EBV. Results revealed that women who were high stress reactors were characterized by higher antibody titers to the latent virus than low stress reactors. High reactors tended to show larger stress-related increases in cortisol than low reactors, but the differences were not significant. Daily stressors can activate the autonomic nervous system and promote the release of pituitary and adrenal hormones, especially in high reactors. Glucocorticoid hormones have been shown to reactivate EBV *in vitro* from cells latently infected with the virus. We hypothesized that absolute levels of plasma cortisol may not be the only explanation for stress-induced reactivation of latent EBV and that the diurnal changes in the production of cortisol may be an important factor in these interactions. To examine the feasibility of this hypothesis, an *in vitro* study was conducted (Study 2) to determine whether changing glucocorticoid concentrations in the medium, in which EBV latently infected cells were cultured, to mimic diurnal changes in plasma cortisol concentrations would enhance the reactivation of the latent virus. Cells latently infected with EBV were exposed to either constant or varying concentrations of the synthetic glucocorticoid hormone dexamethasone (Dex), for 72 h. Results revealed a three- to eightfold enhancement of reactivation of latent EBV in cells pulsed with varying Dex concentrations when compared with cells exposed to a constant and/or a higher mean level of one Dex concentration. Together, these studies raise the possibility that differences in the kinetics of glucocorticoid concentrations may contribute to differences in the reactivation of latent EBV.

Considerable evidence has accumulated linking daily stress levels with the appearance, duration, and intensity of herpesvirus infections and the modulation of the steady-state expression / reactivation of latent Epstein–Barr virus (EBV) (Glaser, Rice, Sheridan, *et al.*, 1987; Glaser and Kiecolt-Glaser, 1994; Kasl, Evans, and Niederman, 1979; Kemeny, Cohen, and Zegens, 1989; Schmidt, Zyzanski, Ellner, Kumar, and Arno, 1985; Stout and Bloom, 1986). The competence of the cellular immune response is a critical factor in controlling primary herpesvirus infections, including EBV, and modulating the expression of the virus in latently infected cells (Glaser and Kiecolt-Glaser, 1994). Patients who are severely immune-suppressed are at risk for severe illness due to reactivation of latent EBV (Gray, Wreghitt, Pavel, *et al.*, 1995). When EBV is reactivated, antibody levels to the virus increase, reflecting the response of memory B lymphocytes to the increase in viral proteins. While counterintuitive, this increase is a negative marker of a deficiency in the cellular immune response.

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Psychological stressors can activate the autonomic nervous system (fight-or-flight response) and promote the release of pituitary and adrenal hormones (McCann, Sternberg, Lipton, Chrousos, Gold, and Smith, 1998). These and other hormones and neuropeptides play an important role in the modulation of the immune system (Munck, Guyre, and Holbrook, 1984) and are thought to be an important gateway through which psychological stressors affect the cellular immune response (Ader, Felten, and Cohen, 1991). For instance, the autonomic innervation of lymphoid organs, together with adrenergic (Madden, Thyagarajan, and Felton, 1998) and glucocorticoid (Bauer, 1983) receptors on immune cells, provide ample avenues through which the nervous and endocrine systems are thought to exert influence on cellular immune function.

Studies from several laboratories have demonstrated increases in antibody titers to latent EBV associated with psychological stress. Using an academic stress model with medical students, we measured EBV antibody titers over time, both at baseline periods in which the students were less stressed and during examination periods when the medical students reported more stress. We found reliable changes in antibody titers to Epstein–Barr virus capsid antigen (VCA) concomitant with the downregulation of several components of the cellular immune response (Glaser *et al.*, 1987; Glaser, Pearson, Jones, *et al.*, 1991; Kiecolt-Glaser, Garner, Speicher, Penn, and Glaser, 1984; Glaser, Pearson, Bonneau, Esterling, Atkinson, and Kiecolt-Glaser, 1993).

In an early study, Kasl and colleagues (1979) followed cadets at West Point, over a 4-year period, who were seronegative for EBV at entry into the Academy. Consistent with the notion that stress can downregulate the cellular immune response and adversely affect the body's ability to respond to infection with EBV, they found that poor academic performance, high levels of motivation for a military career, and an overachieving father were associated with a greater risk for seroconversion to EBV, longer hospitalization in the infirmary following infection (i.e., more severe illness episodes), and higher EBV antibody titers among those who seroconverted but had no clinical symptoms.

B lymphocytes transformed by EBV express glucocorticoid receptors, and glucocorticoid hormones can directly reactivate latent EBV *in vitro* and enhance the lytic replication of the virus. The level of reactivation of latent EBV by dexamethasone (Dex) is generally low, ranging from 2–3% to approximately 20–30% (Bauer, 1983; Glaser, Kutz, MacCallum, and Malarkey, 1995; Joncas & Leyritz, 1974; Magrath, Pizzo, Novikovs, and Levine, 1979). Glucocorticoid hormones are among the hormones modulated by psychological stressors. It is possible that glucocorticoid hormones can directly reactivate latent EBV *in vivo* independent of (or concomitant with) their immune modulatory capacities.

In a previous study, we assessed antibody titers to latent EBV and their relationship to basal plasma cortisol levels. Blood samples were obtained over two time points using pooled hourly samples and designated day- and nighttime samples (Glaser, Pearl, Kiecolt-Glaser, and Malarkey, 1994). We found no relationship between reactivation of latent EBV as defined by changes in antibody titers to EBV and daytime or nighttime levels of cortisol in the same participants (Glaser *et al.*, 1994). Limited *in vivo* samples of cortisol may not be a practical approach to investigating the relationship of plasma cortisol and changes to the steady-state expression of latent EBV as measured by changes in antibody titers. Immunoglobulin G (IgG) has a half-life of approximately 20 days, making any correlation with serum cortisol levels over a few days difficult to interpret.

It is possible that other stress hormones (e.g., catecholamines, corticotropin-releasing hormone (CRH), somatostatin) modulated by psychological stressors might play a role in the reactivation/replication of latent EBV. To test this hypothesis, we conducted an *in vitro* study using an EBV latently infected Burkitt lymphoma (BL) cell line, Daudi. We confirmed previous reports that glucocorticoid hormones can reactivate latent EBV *in vitro* and can also enhance the lytic replication of the virus in cells superinfected with infectious EBV (Glaser *et al.*, 1995). We also found that somatostatin, CRH, and adrenocorticotropin (ACTH) can enhance the lytic replication of EBV; however, we found no evidence that they could reactivate the latent EBV genome in the Daudi cells (Glaser *et al.*, 1995). Of interest is the fact that incubation of the Daudi cells with norepinephrine and epinephrine did not reactivate latent EBV, suggesting that glucocorticoids were more important *in vivo* in promoting the stress-induced reactivation of EBV (Glaser *et al.*, 1995).

We and others have examined cardiovascular reactivity following brief psychological stressors and have found that high and low cardiovascular reactors have different neuroendocrine and immune responses including cortisol reactivity (Lovallo, Pincomb, Brackett, and Wilson, 1990; Sgoutas-Emch, Cacioppo, Uchino, *et al.*, 1994). Subsequent research using autonomic block-

ades (Bernston, Cacioppo, Binkley, Uchino, Quigley, and Fieldstone, 1994; Cacioppo, Uchino, and Berntson, 1994b) and noninvasive indices (Cacioppo, Malarkey, Kiecolt-Glaser, *et al.,* 1995; Uchino, Cacioppo, Malarkey, and Glaser, 1995) revealed that individuals whose cardiovascular reactions to stressors reflect greater activation of the sympathetic branch of the autonomic nervous system tend also to be characterized by larger or more frequent cortisol responses to the stressors. The autonomic blockade research also demonstrated that: (a) the sympathetic activation of the heart could be indexed noninvasively, within specified paradigms, by the preejection period (PEP), which is the time between the electrical signal to the ventricles initiating contraction and the opening of the aortic valve to eject blood from the left ventricle (larger decreases in PEP are associated with greater increases in the *sympathetic* activation of the heart); and (b) the vagal control of the heart could be indexed by respiratory sinus arrhythmia (RSA), which reflects the oscillation in heart period due to the respiratory cycle (larger increases in RSA are associated with greater increases in the *vagal* activation of the heart) (Berntson *et al.,* 1994; Cacioppo, Bernston, Binkley, Quigley, Uchino, and Fieldstone, 1994a). We have suggested that individuals who show high, relative to low, cardiac sympathetic reactivity to laboratory stressors (i.e., larger PEP decreases) also tend to show larger releases of pituitary and adrenal hormones in their daily lives (Cacioppo *et al.,* 1994a; Uchino *et al.,* 1995).

In the first part of this study (Study 1), we performed a secondary analysis of an existing data set from a study of the effects of psychological stressors on autonomic and immune response. In this analysis, we measured serum antibody titers to latent EBV in a sample of older women characterized by high versus low cardiac sympathetic reactivity to brief laboratory stressors involving a degree of personal threat. We hypothesized that high cardiovascular reactivity would be related to the steady-state expression of latent EBV (as measured by antibody titers to the virus) and that this interaction would be mediated by tonic serum cortisol levels. In Study 2, we tested the hypothesis that varying concentrations of the synthetic glucocorticoid hormone, Dex, in the tissue culture medium would induce a higher level of reactivation of latent EBV than in cells treated with a single high concentration of Dex. This *in vitro* study was a follow-up of Study 1 to examine the feasibility of the hypothesis that the kinetics of plasma cortisol concentrations would modulate the reactivation of the latent virus.

METHODS

Study 1

Participants and Procedure

The sample consisted of 50 elderly women (M_{age} = 67.56 years, SEM = 1.13) who were participating in a larger, longitudinal study. The research was conducted following review and approval by the appropriate institutional review board at the Ohio State University. Participants were paid $75.00 for 3.5–4.0 h of participation in this study. All participants were healthy, normotensive, and free of any prescription medication that affects the cardiovascular system. Participants were asked to refrain from ingesting antiinflammatory agents, antihistamines, or alcohol during the 24 h preceding the test day. In preparation for the study, participants were asked: (a) not to consume any alcohol or take any nonprescription medication (e.g., antihistamines) the day before the study, (b) to refrain from exercise the day before the study, and (c) to refrain from eating or drinking anything besides water from midnight on to the time of their scheduled appointment. High reactors were defined as individuals characterized by a large sympathetic reaction to the psychological stressor, as indexed by a median split on stress-induced changes in cardiac PEP, whereas low reactors were defined as those individuals characterized by a relatively small sympathetic reaction to the psychological stressor (Cacioppo *et al.,* 1995). All participants were tested at approximately the same time in the morning.

When participants arrived, the tasks and measures were reviewed, participants' questions were answered, and informed consent was obtained. A serum sample was obtained to measure EBV VCA antibody titers, an occluding cuff of appropriate size was placed over the brachial artery of the other arm for continuous blood pressure measurements, a strain-gauge respirometer was placed midway between the abdomen and thorax, and spot electrodes for impedance cardiography were attached. To allow adaptation to the laboratory, participants were seated upright, given an innocuous set of questionnaires on which to work for approximately 20 min, and asked to sit quietly and relax for 10 min. Following this adaptation period, participants were instructed to sit quietly and relax for 6 min, during which time baseline measures of cardiorespiratory activity were measured. A blood sample was collected immediately following this period for neuroendocrine assessments. Participants next were

exposed to a set of acute laboratory stressors (public speaking and serial subtraction) that lasted 12 min as described in detail in previous reports (Cacioppo *et al.,* 1995). Immediately afterward, another blood sample was collected for neuroendocrine assessments.

In the math stressor, participants performed six 1-min serial subtraction problems continuously for 6 min. Participants were instructed that any error they made would be corrected by the experimenter, and that they should continue from the correct number. The minuend for Min 1 was 297, for Min 2 was 688, for Min 3 was 955, for Min 4 was 593, for Min 5 was 1200, and for Min 6 was 1741. Results from our prior research on mental arithmetic in older adults indicated that participants average approximately 10 serial subtractions per minute (see Cacioppo *et al.,* 1995, for details). The subtrahend in Min 1 was 3. To maintain maximal task involvement and moderate task difficulty (i.e., approximately 10 correct answers per min), the subtrahend specified for each subsequent minute was contingent on the participant's performance during the preceding minute.

In the speech stressor, participants were asked to imagine that they were in a department store shopping when a security guard falsely accused them of shoplifting (Saab, Matthews, Stoney, and McDonald, 1989). Participants were instructed to prepare a 3-min speech to: (a) tell their side of the story, (b) tell the manager what the security guard did wrong and why the security guard may have suspected them of shoplifting, (c) say how they can prove they did not steal the item, (d) specify what should happen to the security guard for the mistake, and (e) summarize their points. Participants were instructed to give intelligent and well-thought-out answers because their speech would be recorded and compared with the speeches of others. Participants were given 3 min to prepare and 3 min to present their speeches.

Measures

Manipulation checks. Following each task participants used 9-point scales (1 = "not at all," 9 = "extremely") to rate how unpleasant, mentally effortful, and arousing the task was. In addition, participants completed the 6-item version of the Spielberger State Anxiety Inventory (Spielberger, Gorsuch, Lushene, Vagg, and Jacobs, 1983) prior to and following the tasks.

Autonomic assessment. As described elsewhere (Cacioppo *et al.,* 1995), the autonomic measures included heart rate and blood pressure as measures of cardiovascular activation, PEP as a measure of cardiac sympathetic activation, RSA as a measure of the vagal activation of the heart, and respiration amplitude and frequency to ensure differences in RSA were not artifacts of respiratory activity.

Neuroendocrine measures. Assays for epinephrine (EPI), norepinephrine (NE), ACTH, and cortisol were performed using plasma from the blood samples drawn at baseline and after the stressors. Plasma catecholamine (EPI and NE) levels were determined by high-performance liquid chromatography using a Waters system with an electrochemical detector. Alumina was used to extract the samples before they were placed on columns. The sensitivity of this system for EPI is 10 pg/ml, and for NE, 20 pg/ml. The assay has intra- and interassay coefficients of variation of 12% for EPI and 7% for NE, and the internal standard to monitor percentage recovery averaged more than 80%. Plasma ACTH levels were measured using an immunoradiometric method (Nichols Institute, Capistrano, CA). This assay has intra- and interassay coefficients of variation of less than 10% and the sensitivity is 1 pg/ml. Plasma cortisol levels were tested using a fluorescence polarization technique (TDX, Abbott Laboratories, Chicago, IL). This assay has intra- and interassay coefficients of variation of less than 10% (Malarkey, Pearl, Demers, Kiecolt-Glaser, and Glaser, 1995).

The indirect immunofluorescence test. The indirect immunofluorescence (IF) test was used to measure antibody titers to EBV VCA as described elsewhere (Glaser *et al.,* 1987). The BL cell line HR-1 was grown in the laboratory and used to make smears on glass coverslips for the IF test. This cell line is latently infected with EBV and expresses viral antigens, which can be detected using serum from an EBV-seropositive person. It is routinely used in the determination of antibody titers to EBV using serum/plasma from subjects. Acetone-fixed HR-1 cells prepared on glass coverslips were adsorbed with twofold dilutions of serum in phosphate-buffered saline to determine endpoint antibody titers to EBV. The highest dilution of serum that resulted in the detection of at least 1% IF-positive cells was considered the endpoint.

Data Analysis

The autonomic data were ensemble averaged within 1-min epochs, and each waveform was verified or edited to remove artifacts prior to analyses. Mean responses were calculated for each minute for each participant. These minute-by-minute means were av-

eraged over the 6-min baseline and each 6-min stressor to increase reliability. Preliminary analyses indicated no significant order effects, so responses to the math and speech stressors were also aggregated to increase measurement reliability.

Independent *t* tests were used to test two a priori contrasts: (1) a difference in baseline levels of psychological, autonomic, neuroendocrine, and EBV antibody titers for low and high reactors, and (b) a difference in reactivity (prestress subtracted from stress) to the laboratory stressors between low and high reactors. An α criterion of $P < 0.05$ was used to evaluate the null hypothesis. Based on the prior literature and a priori expectations, two-tailed tests were used to evaluate basal activity, while one-tailed tests were used to evaluate whether high reactors responded more strongly to the stressors than low reactors. The degrees of freedom in all analyses were adjusted for measures in which technical problems resulted in incomplete data.

Study 2

A 2 (Condition: Constant or Refreshed Concentration) $\times$ 4 (Concentration Level: No Dex, 10^{-9} M Dex, 10^{-7} M Dex, 10^{-5} M Dex) + 1 (10^{-5} M, 10^{-7} M, 10^{-9} M Dex) experimental design was employed. Daudi cells were maintained in the laboratory. Dex has commonly been used by our laboratory and others as a stress hormone to study EBV reactivation *in vitro*. Therefore, logarithmically growing cultures of Daudi cells were prepared at a concentration of 3.5×10^5 cells/ml (>90% viability), then incubated in complete RPMI-1640 medium at 37°C supplemented with 10% fetal bovine serum and containing one of the following nine treatments with Dex: (a) 0 M Dex (medium control) maintained for 3 days (72 h), (b) 1×10^{-9} M Dex maintained for 3 days without changing the medium, (c) 1×10^{-7} M Dex maintained for 3 days without changing the medium, (d) 1×10^{-5} M Dex maintained for 3 days without changing the medium, (e) 0 M (medium control) changed at 8 AM, 12:30 PM, and 5 PM each day for 3 days, (f) 1×10^{-9} M Dex changed at 8 AM, 12:30 PM, and 5 PM each day for 3 days, (g) 1×10^{-7} M Dex changed at 8 AM, 12:30 PM, and 5 PM each day for 3 days, (h) 1×10^{-5} M Dex changed at 8 AM, 12:30 PM, and 5 PM each day for 3 days, and (i) 1×10^{-5} M Dex at 8 AM, 1×10^{-7} M Dex at 12:30 PM, and 1×10^{-9} M Dex at 5 PM each day for 3 days. In all conditions when the medium was changed at 8 AM, 12:30 PM, and 5 PM, the cells were washed and incubated in medium without hormone for 30 min at 37°C to allow the cells to recover before continuing the incubation in medium containing Dex. The cells were finally washed with phosphate-buffered saline, fixed in acetone, and examined by IF to determine the percentage of antigen-positive cells using precharacterized EBV-positive serum as a reflection of the reactivation of the latent virus genome in four independent experiments. A factorial analysis of variance and Scheffe's pairwise comparisons were used, and an α criterion of $P < 0.05$ was used to evaluate the null hypothesis.

RESULTS

Preliminary Tests

The laboratory stressors were designed to be mild and comparably and moderately engaging for all participants. Consistent with this design, high and low reactors expressed comparable basal levels in state anxiety and changes in state anxiety following exposure to the laboratory stressor, and rated the laboratory stressor as equally unpleasant, mentally effortful, and fearful, all P's > 0.05.

Autonomic and Neuroendocrine Activity

Group means are summarized in Table 1. High and low cardiac sympathetic reactors were defined based on a median split on PEP reactivity, $t(46)$, 5.23, $P < 0.001$. Analyses further revealed that high, compared with low, cardiac sympathetic reactors showed larger increases in heart rate, $t(46) = 2.30$, $P < 0.02$, systolic blood pressure, $t(38) = 1.73$, $P < 0.05$, and diastolic blood pressure, $t(38) = 1.79$, $P < 0.04$, as would be expected. Replicating prior research (e.g., Cacioppo, Uchino, and Berntson, 1994), no other autonomic differences were found (see Table 1).

The only neuroendocrine measure to differentiate high and low reactors was basal EPI level, $t(44) = 2.13$, $P < 0.04$, with high reactors characterized by higher circulating EPI levels than low reactors (see Table 1). While high reactors tended to show larger stress-related increases in cortisol ($M = 3.44$) than low reactors ($M = 1.66$), this difference, although in the predicted direction, did not achieve statistical significance, $t(46) = 1.46$, $P < 0.08$. Cortisol assays based on single blood draws before and after a brief stressor have been criticized for their questionable reliability and their sensitivity to irrelevant factors

TABLE 1
Autonomic Response in High and Low Reactors as a Function of Acute Psychological Stressor

	Baseline		Reactivity	
Measure	Low reactors	High reactors	Low reactors	High reactors
PEP	97.28 ± 3.51[a]	93.40 ± 4.68[a]	0.82 ± 0.90[a]	−15.27 ± 2.73[b]
	(n = 24)	(n = 26)	(n = 24)	(n = 26)
HR	66.68 ± 2.29[a]	64.93 ± 1.85[a]	6.90 ± 1.09[a]	11.21 ± 1.54[b]
	(n = 24)	(n = 26)	(n = 24)	(n = 26)
RSA	5.46 ± 0.28[a]	5.47 ± 0.24[a]	−0.10 ± 0.15[a]	−0.37 ± 0.22[a]
	(n = 24)	(n = 26)	(n = 24)	(n = 26)
SBP	139.19 ± 4.24[a]	137.33 ± 4.10[a]	2.05 ± 4.21[a]	12.76 ± 3.79[b]
	(n = 21)	(n = 21)	(n = 21)	(n = 21)
DBP	81.43 ± 2.50[a]	82.00 ± 2.33[a]	−2.76 ± 1.91[a]	2.86 ± 2.33[b]
	(n = 21)	(n = 21)	(n = 21)	(n = 21)
Epinephrine	19.96 ± 1.34[a]	26.21 ± 2.73[b]	6.21 ± 1.77[a]	11.71 ± 3.37
	(n = 24)	(n = 24)	(n = 24)	(n = 24)
Norepinephrine	586.70 ± 45.44[a]	606.76 ± 55.54	−23.70 ± 16.19[a]	10.04 ± 26.59[a]
	(n = 23)	(n = 25)	(n = 23)	(n = 25)
ACTH	14.16 ± 2.14[a]	10.75 ± 1.34[a]	5.67 ± 1.63[a]	7.05 ± 1.86[a]
	(n = 21)	(n = 25)	(n = 21)	(n = 25)
Cortisol	11.42 ± 0.74[a]	9.40 ± 0.78[a]	1.66 ± 0.94[a]	3.44 ± 0.81[b]
	(n = 24)	(n = 26)	(n = 24)	(n = 26)

Note. Mean (±SEM) baseline values (first two columns) and mean (±SEM) change (posttest–pretest) scores (last two columns). Baseline means with differing superscripts and reactivity means with differing superscripts differ statistically at $P < 0.05$. PEP, preejection period in milliseconds (smaller numbers indicate greater sympathetic cardiac activation); HR, heart rate in beats per minute; RSA, respiratory sinus arrhythmia in log units (larger numbers indicate greater vagal cardiac activation); SBP, systolic blood pressure in mm Hg; DBP, diastolic blood pressure in mm Hg. Plasma epinephrine and norepinephrine concentrations are in pg / ml, ACTH in pg / ml, and plasma cortisol concentrations in μg / dl.

such as circadian rhythm. The measurement error associated with differences scores based on single assays may therefore have rendered this assessment insensitive.

EBV VCA Antibody Titers

Results of the contrasts of antibody titers to EBV VCA in high and low reactors confirmed that high reactors showed higher EBV antibody titers (M = 1:1240.62) than low reactors (M = 1:413.33), $t(43) = 2.27$, $P < 0.03$. The results suggest that these differences reflect significant changes in the status of the control over the expression of latent EBV in high and low reactors.

Modulation of Latent EBV by DEX *in Vitro*

The results of Study 1 show that individuals who respond to psychological stressors with large increases in cardiac sympathetic activation are also characterized by having higher antibody titers to latent EBV, suggesting some downregulation of the cellular immune response. Prior research using acute psychological stressors has suggested that high cardiac sympathetic reactors also show elevated stress-induced changes in glucocorticoid hormones (Al'Absi, Bongard, Buchanan, Pincomb, Licinio, and Lovallo, 1997; Cacioppo *et al.*, 1995; Lovallo *et al.*, 1990; Sgoutas-Emch *et al.*, 1994; Uchino *et al.*, 1995). This effect was not statistically significant in the present study, but the threshold is lower for eliciting autonomic than cortisol responses. The laboratory stressor may have been sufficiently evocative to elicit typical autonomic but not cortisol differences to daily stressors. Gauging stress-induced cortisol responses using discrete *in vivo* measures may be insensitive (Schommer, Kudielka, and Helhammer, 1999), which could have also contributed to the nonsignificant difference in cortisol reactivity in the current study. We measured IgG antibody titers to determine the steady-state expression of EBV in Study 1, and IgG has a half-life of 20 days. Thus, the VCA titers we measured in Study 1 could not have been affected by the laboratory stressor. Instead, we speculate that the individual differences in sympathetic reactivity measured in the laboratory reflect individ-

ual differences in stress (including HPA) reactivity generally.

Given the large tonic changes in cortisol over the course of a day, a theoretical implication of the hypothesis that changes in free cortisol in response to psychological stressors can have an impact on the reactivation of latent EBV is that phasic changes in glucocorticoid levels over time can have a larger impact on the reactivation of latent EBV than larger but unchanging (tonic) serum levels of glucocorticoids. To examine the plausibility of this hypothesis, we conducted an *in vitro* study (Study 2), in which the level and kinetics of Dex could be controlled. Since we could not duplicate the exposure of Daudi cells to the exact conditions found *in vivo*, i.e., pulsating diurnal levels of a glucocorticoid hormone, we tested the hypothesis that varying the concentration of Dex over time would modulate the expression of the latent EBV genome differently than exposing cells to a single concentration.

Group means and standard errors for the 2 (Condition: Constant or Refreshed Concentration) $\times$ 4 (Concentration Level: 0, 10^{-9} M Dex, 10^{-7} M Dex, 10^{-5} M Dex) + 1 (10^{-5} M, 10^{-7} M, 10^{-9} M Dex) experimental design are displayed in Fig. 1. The main effect for concentration Level, $F(1, 24) = 41.01$, $P < 0.0001$, confirms previous reports from our laboratory and others that Daudi cells grown in medium only or medium containing 10^{-9}, 10^{-7}, or 10^{-5} M Dex showed 2.25, 2.75, 9.13, and 13.25% EBV antigen-positive cells, respectively. In addition, the main effect for Condition was significant, $F(1, 24) = 8.69$, $P < 0.007$, showing that changing the medium containing a single concentration of Dex three times per day for 3 days produced higher levels of IF-positive cells (8.06%) than maintaining cells in medium containing Dex without change for 3 days (5.63%). The interaction did not approach significance.

As further hypothesized, Daudi cells exposed to varying concentrations of Dex (10^{-5}, 10^{-7}, or 10^{-9} M) for 3 days were found to have 24.00% EBV antigen-positive cells ($P < 0.0001$, pairwise comparisons with each cell of the factorial, see Fig. 1). The percentage antigen-positive cells was higher in this condition than in the conditions in which Daudi cells were exposed to 10^{-5} M Dex alone.

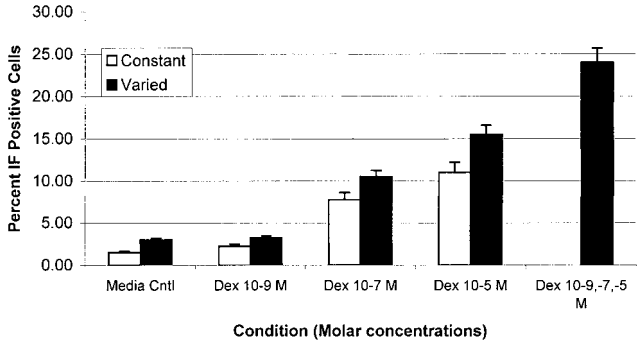

FIG. 1. Mean percentage (SEM) EA/VCA-positive Daudi cells. Daudi cells were prepared at a concentration of 3.5×10^5 cells/ml, then incubated in complete RPMI-1640 medium at 37°C supplemented with 10% fetal bovine serum and containing one of the following nine treatments with dexamethasone (DEX): The experimental conditions were: (a) Medium control (0 M Dex) maintained for 3 days (72 h); (b) medium control (0 M Dex) changed at 8 AM, 12:30 PM, and 5 PM each day for 3 days; (c) 1×10^{-9} M Dex maintained for 3 days without changing the medium; (d) 1×10^{-9} M Dex changed at 8 AM, 12:30 PM, and 5 PM each day for 3 days; (e) 1×10^{-7} M Dex maintained for 3 days without changing the medium; (f) 1×10^{-7} M Dex changed at 8 AM, 12:30 PM, and 5 PM each day for 3 days; (g) 1×10^{-5} M Dex maintained for 3 days without changing the medium; (h) 1×10^{-5} M Dex changed at 8 AM, 12:30 PM, and 5 PM each day for 3 days; and (i) 1×10^{-5} M Dex at 8 AM, 1×10^{-7} M Dex at 12:30 PM, and 1×10^{-9} M Dex at 5 PM each day for 3 days. Each *in vitro* condition was prepared and tested in four independent experiments. All pairwise comparisons were statistically significant ($P < 0.05$).

GENERAL DISCUSSION

The present research produced two new findings. In Study 1, we found, for the first time, that there is a significant difference in the status of the control over the expression/reactivation of latent EBV associated with cardiac sympathetic activation. Data from Study 2, an *in vitro* study, show for the first time that exposing cells latently infected with EBV to phasic changes in Dex levels can enhance the reactivation of latent EBV when compared with a higher but unchanging (tonic) concentration of Dex.

Psychological stress has been implicated as a risk factor in the development, duration, and recurrence of herpesvirus infections. Much of the focus in the literature on EBV latency has been concerned with *in vitro* studies in which B-lymphoblastoid cells and certain epithelial cells latently infected with the virus have been studied (Glaser, Zhang, Yao, *et al.*, 1989; Glaser and Rapp, 1972; Hampar, Derge, Martos, Tagamets, and Burroughs, 1972). Using several different psychological stress models involving normal human participants has allowed us to explore EBV latency from a different perspective, by studying interactions among the central nervous system, the immune system, and the endocrine system, all of which are now known to play roles in the maintenance and control of latent EBV and probably other herpesviruses as well (Glaser and Kiecolt-Glaser, 1994).

In prior studies from our laboratory and others, reliably higher antibody titers to latent EBV were found to be related to an increased level of psychological stress and a downregulation of several aspects of the cellular immune response (Glaser *et al.*, 1987, 1991; Kiecolt-Glaser, Garner, Speicher, Penn, and Glaser, 1984; Esterling, Antoni, Kumar, and Schneiderman, 1990; Sarid, Anson, Yaari, and Margalith, 2001). The elevations in EBV antibody titers in these participants are thought to occur in response to increased synthesis of viral proteins due to reactivation / replication of the latent virus (Glaser and Kiecolt-Glaser, 1994).

Latent EBV can be reactivated *in vitro* by hormones such as Dex and hydrocortisone but not by catecholamines (Bauer, 1983; Glaser *et al.*, 1995; Joncas and Leyritz, 1974; Magrath *et al.*, 1979). Accordingly, we hypothesized that one means by which stress could reactivate latent EBV and enhance virus replication is by increases in cortisol or other stress hormones or neuropeptides. One study that examined the influence of various psychological stressors on cortisol secretion and EBV latency, however, did not find a relationship between cortisol levels and EBV antibody titers. Changes in viral antibody titers are an indirect measure of the efficiency of the cellular immune response in controlling the steady-state expression of latent EBV. In that study, hourly blood samples were obtained and pooled from a baseline period 3–4 weeks before an examination and again during the examination. Examination stress did not elevate serum cortisol levels in this study (Glaser *et al.*, 1994).

The present research raises the possibility that more attention should be paid to phasic changes in or, more generally, to the kinetics of circulating glucocorticoids whether or not groups differ significantly in terms of the magnitude of change. Elevation in a hormone is a feature in the amplitude domain, whereas the kinetics is a feature of the time domain. The quantification and effects of variations in the amplitude and time domains are conceptually orthogonal (cf. Cacioppo and Dorfman, 1987). If the glucocorticoid receptors on, for example, B lymphocytes latently infected with EBV are especially sensitive to phasic changes in cortisol concentrations, then differences in modulation of plasma cortisol levels associated with daily psychological stressors could result in differences in the number of cells in which the latent viral genome is being reactivated. Although it is not possible to simulate an *in vivo* environment to specifically mimic exactly the phasic changes in cortisol, the data from Study 2 showed a three- to eightfold enhancement of reactivation of latent EBV in Daudi cells exposed to varying glucocorticoid concentrations when compared with cells exposed to a constant and higher mean level of one glucocorticoid concentration.

EBV is the etiological agent for infectious mononucleosis (IM), but less than half of people who are infected with EBV actually show clinical symptoms (Henle and Henle, 1979). These data, plus data showing that glucocorticoid hormones can enhance the lytic replication of infectious EBV *in vitro* (Glaser *et al.*, 1995), raise the possibility that the development and severity of EBV-associated IM may be related to everyday stressful events and by phasic glucocorticoid responses to stressors (Kasl *et al.*, 1979).

Since IgG to EBV has a half-life of approximately 20 days, this measure is very stable and does not allow us to link pulsatile changes in serum cortisol over a 1- or 2-day period to EBV antibody titers and, hence, this approach to studying stress-induced changes in viral latency. It is for the above reasons that we attempted to address this question by the *in vitro* Study 2.

In theory, individuals characterized by high, relative to low, cardiac sympathetic reactivity to active coping stressors in the laboratory are also characterized by larger or more frequent activation of the HPA system in their daily life. Second, the differences between high and low reactors in the kinetics of glucocorticoid responses may affect latent EBV *in vivo*. It remains for future research to determine whether the relationship between autonomic function and the steady-state expression of latent EBV (and other latent viruses) is mediated by differences in cortisol reactivity over time in the daily lives of high in contrast to low reactors. Finally, the health implications of the modulation of latent herpesviruses like EBV, cytomegalovirus, herpes simplex virus, and others are not totally understood. While little pathology appears to be associated with the reactivation of latent EBV in normal individuals, there may be significant health risks for individuals who are immune-suppressed (Gray *et al.*, 1995). Thus, the data obtained in this study, along with data from the previous series of studies on stress and EBV reactivation, may provide clues for better models of how the modulation of physiological responses induced by psychological stress can mediate the steady-state expression / replication of latent EBV.

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