

Analysis of Lymphocyte Proliferation Data: Do Different Approaches Yield the Same Results?

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We addressed whether different approaches to the treatment of lymphocyte proliferation data influence the interpretation of results. We focused on two specific issues: (1) the impact of using optimal concentrations of mitogen versus a repeated measures approach using all concentrations and (2) the impact of correcting for unstimulated wells, and the method chosen for doing so. First, analysis of only the optimal concentration yields the same results as using a repeated measures approach. Second, uncorrected stimulated data, difference scores, and statistical control of unstimulated values all yield identical conclusions. The stimulation index, however, frequently yields different outcomes than the other approaches. We discuss reasons for these differences, note that the equivalence between repeated measures and optimal concentration techniques is limited to studies predicting suppression of proliferation irrespective of concentration, and suggest situations when the various approaches to analysis be used. © 1994 Academic Press, Inc

INTRODUCTION

Researchers in psychoneuroimmunology often evaluate cellular immune function by assessing the lymphocyte proliferative response. This assay examines how effectively lymphocytes divide (i.e., proliferate) when stimulated *in vitro* with substances called mitogens. Mitogens are capable of nonspecifically inducing T or B cell proliferation. It is assumed that the more proliferation that occurs, the more effectively cells are functioning. Although this assay is widely used, comparability of studies has been hampered by an inconsistency in the method of data analysis. In this article, we reanalyze data from six studies and compare results from several different statistical strategies. We discuss the similarities and differences between results using each analytic procedure and suggest criteria for choosing an appropriate statistical method.

The proliferation assay involves incubating lymphocytes with a mitogen for several days and then adding a radioactively labeled DNA precursor (tritiated thymidine) to the culture of dividing cells. Each newly formed cell incorporates the isotope into its DNA, and one can quantify the amount of proliferation that occurs by counting the amount of radioactivity taken up by the lymphocytes. Commonly used mitogens include phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM), which preferentially stimulate T cells (PHA and Con A) and B cells (PWM). The proliferative response to mitogens is standardly determined by establishing a dose-response curve by repeating the assay with several concentrations of mitogen. The range of mitogen concentrations is typically chosen to represent the full range of concentrations that reliably stimulate cells in culture. Proliferative response in the *absence* of mitogens (re-

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ferred to as unstimulated proliferation) is also standardly determined. Unstimulated proliferation data serve as an intraassay method of identifying outliers and in some instances, which are discussed below, are used to "correct" stimulated data.

Two traditions have developed with respect to analyzing these data. In one, a repeated measures analysis is used, taking into account all of the concentrations used in the assay. In the other, data from only one of the concentrations is analyzed, specifically the concentration of mitogen that produces the greatest mean proliferative response across all subjects (i.e., the optimal concentration). In addition, data from lymphocytes stimulated with mitogen are often corrected in some fashion with the data from unstimulated lymphocytes. Other times, only data from stimulated wells are used. Moreover, when stimulated data are corrected using unstimulated data, the method of correction varies. For example, unstimulated wells are often taken into account by statistical controls in the analysis or by subtracting them from stimulated data. An approach used less often is to divide unstimulated into stimulated wells, creating what is referred to as a stimulation index.

In this article we address whether these varied approaches to the treatment of lymphocyte proliferation data result in different conclusions. In particular, we focus on two major issues: (1) the impact of using optimal concentrations of mitogen versus a repeated measures approach using all concentrations and (2) the impact of correcting for unstimulated wells, and if they are corrected for, the method chosen for doing so. To do this, we gather data from six studies, analyze it using the different approaches, and compare the results. The data we report come from studies of three species (humans, monkeys, and rats), assess the proliferative response of cells from two immune compartments (blood and spleen), and assess the proliferative response to two mitogens (PHA and Con A).

METHOD

Studies

Below we briefly describe the design of the studies from which we drew data. For more complete descriptions we refer readers to each referenced article. In particular, we analyzed data from six data sets, drawn from four articles published in peer reviewed journals (Bachen, Manuck, Marsland, Cohen, Malkoff, Muldoon, & Rabin, 1992; Cohen, Kaplan, Cunnick, Manuck, & Rabin, 1992; Cunnick, Lysle, Kucinski, & Rabin, 1990; Pezzone, Rush, Kusnecov, Wood, & Rabin, 1992).

Bachen et al. (1992). Forty-four human males (aged 19–25) were randomly assigned to a stress or control condition in a ratio of 3:1. Blood was drawn to assess lymphocyte proliferation in response to PHA before and after subjects were exposed to a 21-min laboratory stressor. A whole blood assay was conducted at concentrations of 0.5, 2.5, 5.0, 10.0, and 20.0 $\mu\text{g/ml}$.

Cohen et al. (1992). Forty-three healthy adult male cynomolgus monkeys were randomly assigned to stable ($n = 22$) or unstable ($n = 21$) social conditions for a 26-month period. Blood was drawn to assess lymphocyte proliferation in response to PHA and Con A for 3 consecutive weeks following the 26-month period. A whole blood assay was conducted at concentrations of 0.5, 2.5, and 5.0 $\mu\text{g/ml}$ of PHA and 2.5, 5.0, and 10.0 $\mu\text{g/ml}$ of Con A.

Cunnick et al. (1990). This paper reported data from three experiments that used male rats of the Lewis strain. The first experiment investigated the effects of adrenalectomy on stress-induced immune alteration (ADX), while the second and third experiments investigated the effects of prior injection of propranolol (PROP) and nadolol (NAD) on stress-induced immune alteration. In all three experiments, subjects were randomly assigned to the relevant control and experimental groups. Experimental subjects were presented with 16 signaled shocks in a single 64-min session. All subjects were then sacrificed and blood and spleen were collected to assess lymphocyte proliferation in response to Con A. Whole spleen assays were conducted at 1.0, 5.0, and 10.0 $\mu\text{g/ml}$ of Con A, and whole blood assays were conducted at 10.0 and 20.0 $\mu\text{g/ml}$ of Con A.

Pezzone et al. (1992). Male rats of the Lewis strain were randomly assigned to one of three conditions consisting of a series of two injections spaced one-half hour apart: saline/saline, saline/amphetamine, and propranolol/amphetamine injections. All subjects were sacrificed 1 h after the second injection and blood and spleen were collected to assess lymphocyte proliferation in response to PHA and Con A. Whole spleen and whole blood assays were conducted at concentrations of 1.0 and 10.0 $\mu\text{g/ml}$ of PHA and Con A.

Data Analysis

All data sets were obtained from the authors and were prepared for analysis in an SPSSX environment. Prior to the data analysis for this paper, we replicated the analyses reported in the published papers to ensure that we were analyzing the correct sets of data. We then analyzed each data set using each of four approaches: (1) uncorrected—using only stimulated wells; (2) covaried—using unstimulated wells as covariates in the analysis; (3) difference scores—subtracting unstimulated wells from stimulated wells; and (4) stimulation index—dividing stimulated wells by unstimulated wells. Each of these four approaches was done using both a repeated measures technique and only the optimal concentration of mitogen. Therefore, a total of eight analyses were conducted with each data set. Optimal concentration was defined as the concentration of mitogen that produced the greatest mean proliferation across all subjects in the study. Optimal concentrations for the data sets were as follows: 5 $\mu\text{g/ml}$ PHA in Bachen et al. (1992); 10 $\mu\text{g/ml}$ Con A and 5 $\mu\text{g/ml}$ PHA in Cohen et al. (1992); 10 $\mu\text{g/ml}$ Con A for spleen and 20 $\mu\text{g/ml}$ Con A for blood in Cunnick et al. (1990); and 5 $\mu\text{g/ml}$ Con A for spleen, 10 $\mu\text{g/ml}$ Con A for blood, and 10 $\mu\text{g/ml}$ PHA for blood and spleen in Pezzone et al. (1992).

Prior to performing any analyses, we investigated whether the lymphocyte proliferation data were normally distributed. Using formulae provided by Bliss (1967), we determined whether the skew, the kurtosis, or the two combined were reliably different from what would be expected from a normal distribution. If departures from normality were encountered, \log_{10} transformations were done. The data transformed prior to analyses include all Bachen et al. (1992) data, the stimulation index for blood PHA and Con A from Cohen et al. (1992), the stimulation index for blood and spleen Con A from the ADX experiment, all blood Con A data from the PROP experiment, the stimulation index for blood Con A and all spleen Con A data from the NAD experiment in Cunnick et al. (1990), and the stimulation index for spleen PHA and Con A from Pezzone et al. (1992). The

formulae provided by Bliss (1967) indicated that all transformed data were normally distributed.

The specific analyses performed were ANOVAs or repeated measures ANOVAs. A main effect or interaction is reported only if at least one of the eight analyses resulted in a reliable F statistic. In any case where a reliable F statistic was found, the F values for all eight analysis approaches were transformed into effect sizes. An effect size indicates how large an association is between two variables, disregarding sample size (Rosenthal, 1984, 1991). We used the product-moment correlation coefficient (r) as the effect size estimate and present the effect sizes to allow for comparison across the different analytic approaches to proliferation data. Effect sizes were compared using tests for differences between correlations from dependent samples (Cohen, 1977). For interested readers we also indicate whether the effect sizes represent a reliable F statistic using the criterion that $p < .05$.

RESULTS

Tables 1 through 4 present the results of the analyses from the Bachen et al. (1992) data, the Cohen et al. (1992) data, the Cunnick et al. (1990) data, and the Pezzone et al. (1992) data, respectively. Each table presents effect sizes for the main effects or interactions where at least one of the eight analyses resulted in a reliable F statistic. In each case, eight effect sizes are presented that represent the analyses that cross multiple vs optimal concentrations with the four methods of correcting for data generated by unstimulated lymphocytes. Below we address each of our questions in turn.

Optimal Concentrations vs Repeated Measures

Of the 92 comparisons it is possible to make, effect sizes from analyses of the optimal concentration differ from the repeated measures approach in only four cases. All of these are from the Cunnick et al. (1990) data (see Table 3), deal with the Stress main effect, and include: the covaried analyses of the ADX blood Con A data and the uncorrected, covaried, and difference analyses of the PROP blood Con A data (all $ps < .05$). In each of those four cases, the effect size from the optimal concentration analysis was smaller than that from the repeated measures

TABLE 1
Effect Sizes for Eight Different Analysis Approaches to the Bachen et al (1992) Lymphocyte Proliferation Data

Blood PHA	Uncorrected		Covaried		Difference		Stimulation	
	Rep	Opt	Rep	Opt	Rep	Opt	Rep	Opt
Group × Time	.457*	.480*	.429*	.460*	.457*	.480*	.113 ^a	.096 ^a
Concentration	.863*		.863*		.863*		.863*	

Note. A main effect or interaction is reported only if at least one of the eight analyses resulted in a reliable F statistic. Rep, analyses using multiple concentrations of mitogen; Opt, analyses using only the optimal concentration of mitogen; Group, subject assignment to stress or control conditions; Time, baseline or poststress; Concentration, concentration of mitogen. Blank spaces indicate effects not tested in the analyses.

* Analysis yielded a reliable F statistic.

^a Reliably different when compared to the uncorrected, covaried, and difference effects.

TABLE 2
Effect Sizes for Eight Different Analysis Approaches to the Cohen et al (1992) Lymphocyte Proliferation Data

	Uncorrected		Covaried		Difference		Stimulation	
	Rep	Opt	Rep	Opt	Rep	Opt	Rep	Opt
Blood Con A								
Affiliation	.314*	.266	.325*	.280	.319*	.270	.118	.087
Stability	.378*	.451*	.395*	.469*	.391*	.460*	.389*	.436*
Time	.371*	.381*	.371*	.381*	.371*	.381*	.132 ^a	.163
Aff × Sta × Time	.210	.224	.200	.218	.203	.220	.363*	.361*
Concentration	.891*		.891*		.891*		.919*	
Sta × Con	.434*		.434*		.434*		.145 ^a	
Time × Con	.348*		.348*		.348*		.143	
Aff × Con	.097		.097		.097		.249	
Blood PHA								
Time	.374*	.205	.375*	.206	.375*	.206	.328*	.331*
Aff × Sta × Time	.136	.146	.117	.113	.127	.138	.296*	.364 ^{a,b}
Concentration	.862*		.862*		.862*		.771 ^a	
Time × Con	.277*		.277*		.277*		.357*	
Aff × Sta × Time × Con	.065		.065		.065		.255*	

Note. A main effect or interaction is reported only if at least one of the eight analyses resulted in a reliable *F* statistic. Rep, analyses using multiple concentrations of mitogen; Opt, analyses using only the optimal concentration of mitogen; Affiliation/Aff, monkey classification as high or low affiliation; Stability/Sta, monkey assignment to stable or unstable social conditions; Time: first, second, or third week of blood drawing; Concentration/Con, concentration of mitogen. Blank spaces indicate effects not tested in the analyses.

* Analysis yielded a reliable *F* statistic.

^a Reliably different when compared to the uncorrected, covaried, and difference effects.

^b Reliably different when compared to the covaried effect.

analysis. In the remaining 88 cases, effect sizes for the repeated measures analyses do not differ from effect sizes from analyses using only the optimal concentration of mitogen.

Correcting Stimulated with Unstimulated Data

We compared whether effect sizes generated from uncorrected data differed from those generated using the other three approaches. Analyses using uncorrected data never differ reliably from those using difference scores or those that covary the unstimulated data. On the other hand, the stimulation index differs from the other methods of analyses in 22 of a possible 54 cases. First, from the Bachen et al. (1992) data, the effect size for the Group × Time interaction is reliably lower using the stimulation index analyses than it is for the uncorrected, covaried, or difference score analyses ($p < .01$) (see Table 1). Results from the Pezzone et al. (1992) data indicate that the stimulation index analysis of spleen PHA produces a larger effect size for Concentration than the other three approaches ($p < .01$) (see Table 4).

Data from Cohen et al. (1992) show that the stimulation index differs for Con A as well as PHA (see Table 2). With respect to Con A, the repeated measures stimulation index analyses results in smaller effect sizes for Time ($p < .05$) as well as for Stability × Concentration ($p < .01$), when compared to the values obtained

TABLE 3
Effect Sizes for Eight Different Analysis Approaches to the Cunnick et al. (1990) Lymphocyte Proliferation Data: The ADX, PROP, and NAD Experiments

	Uncorrected		Covaried		Difference		Stimulation	
	Rep	Opt	Rep	Opt	Rep	Opt	Rep	Opt
<u>ADX Blood Con A</u>								
Stress	.798*	.718*	.759* ^c	.642* ^c	.799*	.719*	.839*	.852* ^a
Operate	.597*	.599*	.597*	.601*	.597*	.598*	.566*	.546*
Op × Str	.337	.333	.336	.324	.338	.333	.492*	.497*
Concentration	.067		.067		.067		.374* ^a	
Str × Con	.199		.199		.199		.337*	
<u>PROP Blood Con A</u>								
Stress	.766* ^c	.610* ^c	.712* ^c	.529* ^c	.758* ^c	.607* ^c	.806* ^{ab}	.833* ^{aa}
Concentration	.771*		.771*		.747*		.771*	
Inj × Con	.458*		.458*		.429*		.458*	
Str × Con	.659*		.659*		.643*		.659*	
Str × Inj × Con	.497*		.497*		.421*		.497*	
<u>NAD Blood Con A</u>								
Stress	.893*	.871*	.850*	.814*	.893*	.871*	.859*	.819*
Str × Inj	.451*	.420*	.433*	.396	.451*	.420*	.444*	.406
Concentration	.947*		.947*		.947*		.889* ^a	
Str × Con	.556*		.556*		.556*		.586*	
<u>ADX Spleen Con A</u>								
Stress	.839*	.855*	.791*	.812*	.838*	.855*	.534* ^{aa}	.577* ^{aa}
Operate	.289	.296	.359	.339	.304	.301	.617* ^{aa}	.554* ^{aa}
Op × Str	.225	.318	.226	.320	.227	.321	.263	.328
Concentration	.888*		.888*		.888*		.921*	
Str × Con	.727*		.727*		.727*		.619*	
Op × Con	.233		.233		.233		.335*	
Op × Str × Con	.217		.217		.217		.341*	
<u>PROP Spleen Con A</u>								
Stress	.538*	.512*	.507*	.495*	.539*	.513*	.454*	.432*
Inj × Str	.424	.402	.447*	.427*	.413	.399	.396	.371
Concentration	.914*		.914*		.914*		.882*	
Str × Con	.491*		.491*		.491*		.443*	
Str × Inj × Con	.380*		.380*		.380*		.384*	
<u>NAD Spleen Con A</u>								
Inject	.724*	.725*	.719*	.712*	.732*	.726*	.736*	.724*
Str × Inj	.538*	.517*	.541*	.513*	.542*	.518*	.523*	.503*
Concentration	.970*		.970*		.971*		.970*	
Inj × Con	.556*		.556*		.462*		.556*	
Str × Inj × Con	.458*		.458*		.394*		.458*	

Note. A main effect or interaction is reported only if at least one of the eight analyses resulted in a reliable F statistic. Rep, analyses using multiple concentrations of mitogen; Opt, analyses using only the optimal concentration of mitogen; Stress/Str, rat assignment to stress or control conditions; Operate/Op, rat assignment to adrenalectomy, sham adrenalectomy, or control conditions; Concentration/Con, concentration of mitogen; Inject/Inj, concentration of propranolol or nadolol the rat received. Blank spaces indicate effects not tested in the analyses.

* Analysis yielded a reliable F statistic.

^a Reliably different when compared to the uncorrected, covaried, and difference effects

^b Reliably different when compared to the covaried effect.

^c Reliable differences between repeated measures and optimal concentration analyses.

from the other three approaches. In terms of PHA, the stimulation index again results in a smaller effect size for Concentration ($p < .05$) when compared to the other three approaches. In addition, the optimal concentration stimulation index effect size is larger than the covaried value for Affiliation × Stability × Time ($p < .05$).

Data from Cunnick et al. (1990) show that the stimulation index differs for blood as well as spleen Con A (see Table 3). For blood Con A, data from the ADX experiment show that the optimal concentration stimulation index for the Stress

TABLE 4
Effect Sizes for Eight Different Analysis Approaches to the Pezzone et al. (1992) Lymphocyte Proliferation Data

	Uncorrected		Covaried		Difference		Stimulation	
	Rep	Opt	Rep	Opt	Rep	Opt	Rep	Opt
<u>Blood Con A</u>								
Group	.711*	.768*	.710*	.776*	.711*	.701*	.479	.573*
Concentration	.871*		.871*		.871*		.845*	
Grp × Con	.820*		.820*		.820*		.743*	
<u>Blood PHA¹</u>								
Group		.723*		.750*		.722*		.659*
<u>Spleen Con A¹</u>								
Group		.820*		.859*		.826*		.885*
<u>Spleen PHA</u>								
Group	.961*	.963*	.975*	.974*	.965*	.949*	.951*	.952*
Concentration	.983*		.983*		.983*		.993* ^a	
Grp × Con	.955*		.955*		.955*		.943*	

Note. A main effect or interaction is reported only if at least one of the eight analyses resulted in a reliable *F* statistic. Rep, analyses using multiple concentrations of mitogen; Opt, analyses using only the optimal concentration of mitogen; Group/Grp, animal assignment to a particular injection protocol condition; Concentration/Con, concentration of mitogen. Blank spaces indicate effects not tested in the analyses.

¹ Only one concentration of mitogen was assessed across all animals, so only the optimal concentration analysis was performed.

* Analysis yielded a reliable *F* statistic.

^a Reliably different when compared to the uncorrected, covaried, and difference effects.

main effect results in a larger effect than the covaried analysis, and the repeated analysis stimulation index of Concentration also results in a larger effect size ($p < .01$). Data from the PROP experiment show that the optimal concentration stimulation index for the Stress main effect results in a larger effect than the other approaches ($p < .01$). In addition, the repeated measures stimulation index for the Stress main effect results in a larger effect size when compared to the covaried value ($p < .05$). Finally, data from the NAD experiment show that the repeated stimulation index effect size for Concentration is smaller than the other three approaches ($p < .01$). In terms of spleen Con A data, the ADX experiment shows that the stimulation index effect sizes for Stress and Operate are smaller and larger, respectively, than those found using the other methods of analysis ($ps < .05$).

DISCUSSION

We addressed two specific issues with respect to how the analytic treatment of lymphocyte proliferation data might have an impact on conclusions drawn. First, we determined the influence of using only the optimal concentration of mitogen versus a repeated measures approach using all concentrations. Regardless of the approach, the outcomes, and therefore the conclusions that would be drawn, are the same. This is true for data from humans, monkeys, and rats, for blood lymphocytes and spleen cells, and for PHA and Con A. In terms of the effect sizes, the repeated measures analyses only differed from the optimal concentration anal-

yses in 4 of a possible 92 comparisons. This number of differences is no more than would be expected by chance, given the total number of comparisons made. Thus, when main effects of stress are compared, both approaches yield the same conclusions.

The second primary issue we investigated in this article was the impact of correcting for unstimulated wells, as well as the impact of the method chosen for doing so. In general, results of the analyses indicate that using only uncorrected stimulated wells, difference scores, or statistically controlling the unstimulated data yield identical conclusions. The discrepancies that arise are all in connection with the stimulation index. Specifically, of 54 possible main effects or interactions, the effect sizes generated using the stimulation index differ from the other methods of analysis in 12 cases (22.2%). The direction of difference is not uniform, with half the effects being larger and half smaller than the other methods. It has been pointed out by others that the stimulation index is a problematic indicator of lymphocyte proliferation because, since it is a ratio, it weighs unstimulated values so heavily (Maluish & Strong, 1986). The problem is that unstimulated values vary a great deal for a number of reasons other than the intrinsic ability of cells to incorporate tritiated thymidine. Because the stimulation index appears to magnify the error inherent in the assay, we agree with Maluish and Strong (1986) and recommend that it not be used.

In terms of the other three approaches to analysis, our data indicate that regardless of whether the unstimulated wells are taken into account, the outcome is the same for the range of concentrations used in these studies. This is because the variation in the unstimulated wells is relatively trivial, and neither difference scores nor using statistical controls weighs the variation very heavily. Thus, in cases such as these the most straightforward approach to data analysis would be to use only the data generated by stimulated lymphocytes. However, if the researcher chooses to account for the proliferation generated by unstimulated lymphocytes, taking these data into account does not appear to produce qualitatively distinct conclusions.

The studies we examined all hypothesized stress-induced suppression of proliferation, and the conclusion that optimal concentration and repeated measures analyses are equivalent should be limited to such studies. Implied in the stress-induced suppression hypothesis is that stress effects on proliferation will be relatively consistent across concentrations of mitogen that stimulate proliferation but are not toxic to cells. Hence, assuming appropriately chosen concentrations, one would expect a main effect of stress in a repeated measures analysis of variance. Such a result indicates that stress is associated with proliferation when you collapse across concentrations. Because the opportunity for demonstrating suppression is greatest in the case of the concentration inducing maximal proliferation (i.e., the "optimal" concentration), the results from examining this concentration alone should be consistent with the results from the repeated measures design. More problematic is when the hypothesis is stress-induced suppression and the use of a repeated measures design indicates a stress by concentration interaction. Such an interaction indicates that there are different relations between stress and proliferation at different concentrations of mitogen. This often occurs in addition to the main effect of stress, but can also occur without a reliable main effect. In this context, the interaction is an unexpected result and should be interpreted cautiously. With appropriately chosen concentrations, the probability

of an interaction occurring by chance is high. Alternatively, an interaction is likely when testing hypothesized immunosuppression if the lowest concentration is too low to actually induce proliferation or the highest concentration is too high and consequently is toxic to the cells. In this case, stress would be associated with decreased proliferation for appropriately chosen concentrations but not for the inappropriate ones.

The mirror image of this issue occurs when the prediction is enhancement of proliferation regardless of concentration. In this case, you would also expect a main effect for stress in a repeated measures analysis. Stress by concentration interactions would again be unexpected, and should be interpreted cautiously. The equivalent single concentration analysis in this case would be a *suboptimal* concentration, rather than the optimal concentration since the opportunity for demonstrating enhancement is greatest in the case of concentrations inducing minimal proliferation.

Finally, those with an *a priori* interest in exploring differential stressor effects at various concentrations face a different situation. For example, under some conditions stressors might facilitate cell division occurring in response to low levels of mitogen, but inhibit or not affect responses to optimal levels. This would result in an interaction in a repeated measures design. Unfortunately, work indicating a stress by concentration interaction has seldom been based on *a priori* hypotheses. In these cases, there is a substantial possibility that different results in response to different concentrations occurred merely by chance. The way to guard against the chance interpretation, of course, is to work from *specific* plausible hypotheses, appropriately adjust the alpha level to reflect the number of tests (concentrations), or be explicit about the exploratory nature of the work. Choosing only an optimal (or suboptimal) concentration in this context makes no sense. Instead, a repeated measures approach to analysis would be necessary, and an interaction between stress and concentration (rather than a main effect of stress) would be predicted in the analysis of variance model.

If a researcher chooses to use the optimal concentration of mitogen for analysis, one final important point is that doing so involves more than just running the assay with a single concentration of mitogen, or analyzing and reporting results from only the concentration of mitogen that best illustrates an effect. The former approach might be problematic if the concentration chosen stimulates lymphocytes either not enough or too much. The latter approach, on the other hand, is unacceptable because it ignores null results and capitalizes on chance by increasing the number of analyses and hence, the probability of finding an effect due to chance. For example, if five concentrations of mitogen are used in an assay and analysis of each separately indicates that only one shows a reliable ($p < .05$) effect, the probability that the particular finding reflects a chance occurrence is substantial (one chance in four). Therefore, we believe that the only acceptable method of choosing a single concentration for analysis is to establish a dose-response curve using at least three mitogen concentrations and to choose the optimal concentration by determining the one that produces the greatest mean proliferation across all subjects.

In sum, we determined that uncorrected stimulated data, as well as difference scores or analysis of covariance, yield identical conclusions in the treatment of lymphocyte proliferation data. Therefore, within these approaches to analysis, investigators appear to be free to choose the method of analysis with which they

feel most conceptually comfortable. The stimulation index, however, does not appear to be an appropriate technique to correct for unstimulated data. We also determined that when suppression of proliferation is hypothesized and there is no reason to expect differences between concentrations, the optimal concentration and repeated measures approaches are equivalent. In studies in which differences between concentrations are expected, however, the optimal concentration is inappropriate and the repeated measures approach is necessary. Overall then, regardless of the purpose of the study, repeated measures analysis of variance is always an appropriate approach to analysis. The primary issue for researchers to guard against, however, is the inappropriate interpretation of unpredicted interactions of stress with concentration.

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