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RELIABILITY OF LYMPHOCYTE PROLIFERATION ASSAYS

LISE FILLION, MPs
University of Miami, Department of Psychology, Behavioral Medicine Research Center, P.O. Box 248185, Coral Gables, FL 33124-2070, USA

MARTHE BELLES-ISLES, PhD
Centre de recherche en Inflammation et Immunologie-Rhumatologie, Le Centre Hospitalier de l'Université Laval, Québec, Canada

LOUISE LEMYRE, PhD
École de psychologie, Université Laval, Canada

RAYNALD ROY, Phd
Centre de recherche en Inflammation et Immunologie-Rhumatologie, Le Centre Hospitalier de l'Université Laval, Québec, Canada

SUMMARY

According to the psychoneuroimmunology literature, stressful experiences and psychological stress can alter various immunological parameters. The lymphocyte proliferation response of T cells is known to be variable but no data can be found on the reliability of blastogenesis retesting. To address this problem, 10 healthy volunteer men were tested on four occasions in longitudinal repeated measures design. Immunological parameters were measured from blood samples testing date. Using a doubly multivariate repeated measures design, significant time and concentration effects were observed for both parameters: concanavalin A induced blastogenesis (ConA) and phytohemagglutinin induced response (PHA). An interaction effect between time and mitogen dose was observed. Satisfactory reliability for ConA could not be achieved even in controlling for mitogen dose. Better results were obtained for PHA, but only at dilution 1:100. These data highlight the need for caution in interpreting fluctuations associated with lymphocyte proliferation responses in psychoneuroimmunology.

KEY WORDS—Psychoneuroimmunology, lymphocyte, PHA, ConA, reliability.

Evidence suggesting that stressful experiences or psychological stress can alter various immunological parameters has accumulated over the last two decades.[1] Despite rapid growth in psychoneuroimmunology (PNI), several fundamental questions remain partly unanswered. Among these, longitudinal designs and repeated measure analyses have been largely used without establishing reliability of immunological data. Most of the time, immune measures have been initially validated for clinical purposes and directly imported from clinical use to PNI research without any adaptation of the assay. The reliability of immune assays used in these studies remains to be demonstrated.

T-lymphocyte mitogens like concanavalin A (ConA) and phytohemagglutinin (PHA) have been widely used to monitor cell-mediated immunocompetence in previous studies of stress and immune function. The lymphocyte proliferation assay is recognized to be particularly sensitive to psychological stress.[2, 3] The effects of natural acute stressors such as bereavement,[3] divorce[4] and academic examinations, [5, 6] chronic stressors such as unemployment[7] and clinical states such as depression[8] have all been associated with suppression of lymphocyte proliferation to PHA or ConA mitogen challenge. More recently, some studies have reported decreases in lymphocyte proliferation to ConA and PHA after exposure to experimental stressors.[9–11]

Radioactive thymidine incorporation into newly synthesizing populations of separated lymphocytes has been the method most often used.[12] This method has been standardized by Fitzgerald.[13] There is considerable controversy over the manner in which proliferation assay data are best expressed. The most common method is to calculate net counts per minute (cpm), the cpm of the stimulated cultures minus the cpm of the unstimulated control cells. According to Fletcher *et al.*,[14] using only the cpm of the stimulated cultures could

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sometimes improve stability because unstimulated cpm could vary considerably in certain patient populations.

In spite of the fact that the lymphocyte blastogenic response is known to vary with cyclic fluctuations such as circadian rhythms,[15] the question of this type of assay's reliability has received little attention in the PNI field. In fact, only Fletcher *et al.*[14] have reported results about PHA proliferation assay reliability.

This study examines the reliability of ConA and PHA lymphoproliferative responses for a 2-month period among 10 health young men.

METHOD

Subjects

Subjects in this longitudinal study were 10 healthy volunteer males aged between 24 and 36 years of age from the laboratory staff. No subjects suffered from illness, used prescription medication or illicit drugs. Blood samples were collected on test days 0, 14, 28 and 42 (time 1 to time 4 (T1–T4)) from April to June. In order to control for circadian variations, all blood samples were collected on Monday mornings between 8.00 and 8.30 am. Subjects were required to fast for 12 hours and to avoid alcohol and caffeine for 24 hours prior to blood sampling. In order to control for psychological stress, subjects completed the Psychological Stress Measurement,[16] an evaluation of the psychological stress state level.

Immunological data: ConA and PHA blastogenesis of lymphocytes

Mononuclear leucocytes were isolated from peripheral blood by density gradient centrifugation (Ficoll Hypaque) and were cultured for 3 days in RPMI 1640, 10 per cent FCS, PSG, at three dilutions of PHA (1/5, 1/50, 1/100) and four concentrations of ConA (5, 15, 30, 60 μg/10^6 cells) to define dose–response curves. Triplicates were conducted for each condition. Results represent the total tritiated thymidine (cpm) incorporated during the last 16 hours of incubation. All assays were performed on the same day as sampled, at the same time, by the same well-trained technician, with identical materials and lot numbers for all reagents used, and kept under the same storage conditions.

STATISTICAL ANALYSES

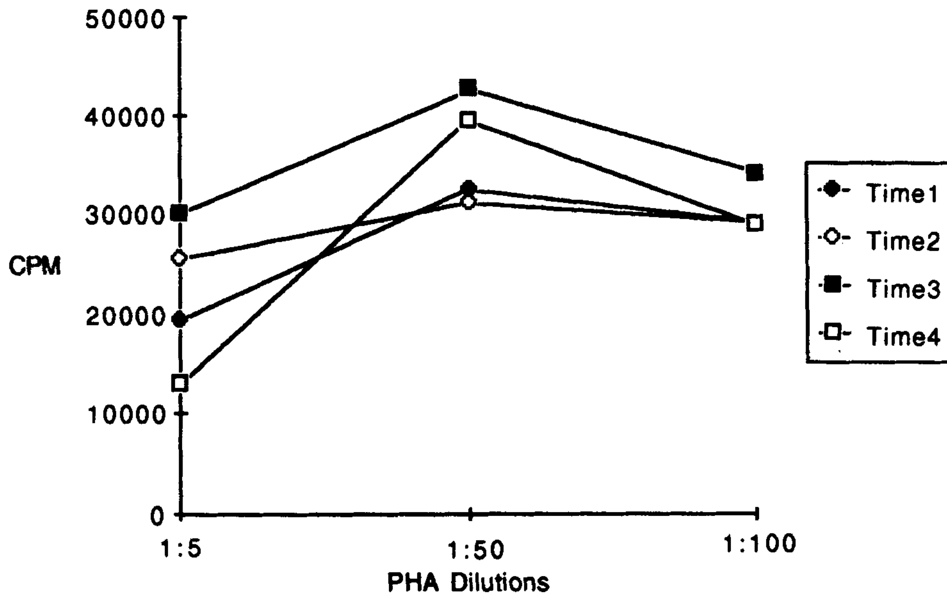
To study stability of the proliferation assay, a doubly multivariate repeated measures design (MANOVA) was used to test for significance of the two within-subject factors: *time* of measurement (T1–T4) and *concentration* within each period. This analysis was followed by repeated measures analysis of variance (ANOVA) to test for significance of *time* for each concentration. In a further effort to estimate reliability of proliferation assays, an extension of generalizability theory (GT) adapted to PNI by Llabre[17] was applied. In GT, the G and G^* coefficients provide an estimate of test–retest reliability. Calculation of G involves computing intraclass rather than interclass correlations and considers multiple sources of variance simultaneously. In GT, the two types of generalizability coefficients (G and G^*) are analogous to reliability coefficients in classical theory. Llabre *et al.*[17] have suggested that a G^* value around 0.80 is indicative of a generalizable measure.

RESULTS

The dose–response curves for PHA and ConA are illustrated in Fig. 1. Maximum stimulation in all 10 subjects was obtained with dilution 1:50 for PHA and concentration 30 μg/ml for ConA.

MANOVA was used to test for the significance of the overall dose–response curve assay results (Table 1). As expected, there was a significant concentration effect for each mitogen (Wilks lambda ($W\lambda$) = 0.008, $p < 0.0001$). Not expected, though, was a time effect for both mitogens ($W\lambda$) = 0.2310, $p < 0.0001$). Significant differences were principally observed for the contrast between T2 and T3 (F (PHA) = 25.63, $p < 0.0001$; F (ConA) = 26.99, $p < 0.0001$). However, there was a significant concentration × time interaction effect between for both PHA and ConA ($W\lambda$ = 0.093, $p < 0.0001$). This interaction effect is illustrated in Fig. 1. PHA responses were more stable for the 1:100 dilution. ConA responses were more stable at the 15 μg/ml and 5 μg/ml concentrations.

In addition to the overall effect as expressed in dose–response curves for PHA and ConA, ANOVAs were performed for each dose for both PHA and ConA, controlling for inflated type I error rate (Table 2). With PHA, the time effect was still significant for proliferation at each dilution except 1:100. For ConA, the time effect was also significant at



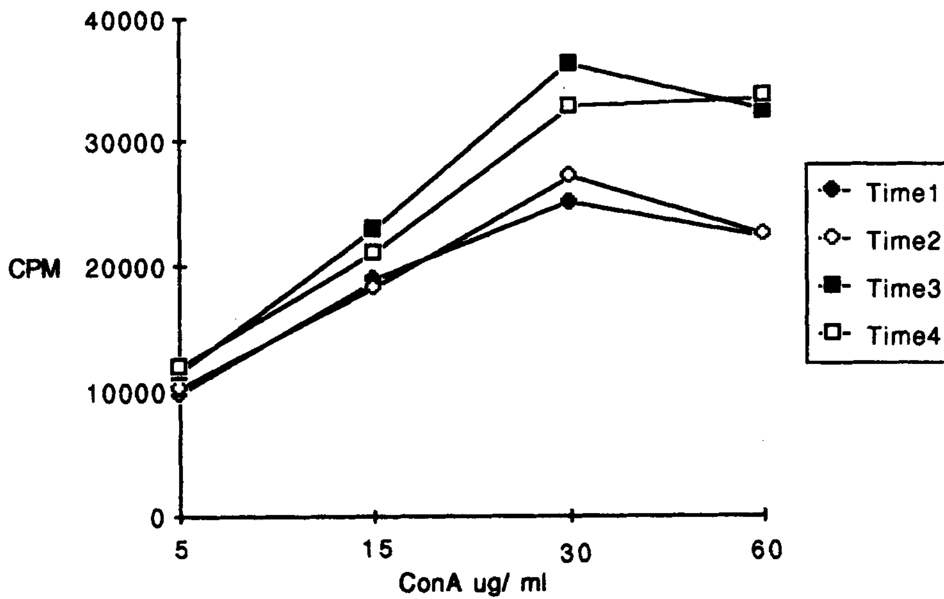


Fig. 1—Dose-response Curves for PHA and ConA

Table 1—MANOVA for PHA and ConA

Effect	Wilks' lambda	*df*	*F*
Concentration	0.008	6	121.45****
Time	0.231	6	17.25****
Concentration × time	0.093	18	6.44****
PHA time contrast			
T1 vs T2			0.46 NS
T2 vs T3			25.63****
T3 vs T4			2.95 NS
ConA time contrast			
T1 vs T2			0.07 NS
T2 vs T3			26.99****
T3 vs T4			0.60 NS

**** $p < 0.0001$.
NS, not significant.

Table 2—Repeated measures analysis of variance for PHA and ConA by concentration

Variable	Means square	*df*	*F*
PHA 1:100	0.0166	3	2.47 NS
PHA 1:50	0.0439	3	9.19***
PHA 1:5	0.3573	3	10.94****
ConA 5 μg/ml	0.0166	3	1.33 NS
ConA 15 μg/ml	0.0259	3	3.08*
ConA 30 μg/ml	0.0607	3	8.51***
ConA 60 μg/ml	0.3613	3	6.33***

* $p < 0.05$.
*** $p < 0.001$.
**** $p < 0.0001$.
NS, not significant

Table 3—Reliability *G* and *G** coefficients and mean Pearson *r* coefficients for PHA (two replications) and ConA (three replications)

Variable	Number of tests	*G*[a]	*G**	Pearson[b] *r* (means)
PHA 1:100	4	0.81	0.78	0.62*
	3	0.83	0.78	
	2	0.71	0.71	
PHA 1:50	4	0.52	0.39	0.31
	3	0.56	0.37	
	2	0.59	0.59	
PHA 1:5	4	0.66	0.50	0.39
	3	0.55	0.46	
	2	0.79	0.68	
ConA 5 μg/ml	4	0.71	0.70	0.48
	3	0.66	0.66	
	2	0.59	0.59	
ConA 15 μg/ml	4	0.51	0.46	0.27
	3	0.47	0.42	
	2	0.55	0.55	
ConA 30 μg/ml	4	0.54	0.40	0.27
	3	0.39	0.26	
	2	0.63	0.62	
ConA 60 μg/ml	4	0.29	0.21	0.15
	3	0.53	0.43	
	2	0.63	0.62	

* $p < 0.05$.
[a]To compute the coefficients (see Llabre *et al.*[17], the model used for the estimate of variance components in these data is $Y = P + T + (R{:}T) + (PT) + (PR{:}T)$ where Y represents the proliferation assay result in log10 cpm, P represents the effect for a person, T represents the systematic effect of the time, $R{:}T$ is the systematic effect of the replication nested within the time, PT is the person × time interaction effect, and $PR{:}T$ is the person × replication interaction.
[b]Mean of Pearson correlations for the four test occasions.

each concentration except 5 μg/ml. Stability of results across time could be achieved for only one dose of each mitogen proliferation assay.

Generalizability theory was applied to further the study of stability. Table 3 displays *G* and *G** coefficients obtained for up to four testing times and for two replications (duplicata) for PHA and for up to four times and four three replications (triplicata) for ConA. The results indicated that only PHA 1:100 with three measurements reached a good criterion of reliability ($G = 0.81$) and generalizability ($G^* = 0.78$). A low-acceptable or borderline criterion of reliability (G and $G^* = 0.710$) could be obtained for PHA at 1:100 dilution with two measurements and for ConA at 5 μg/ml concentration with four measurements. The mean of Pearson correlations for the four testing dates was significant only for PHA at 1:100 dilution ($r = 0.62$, $p < 0.05$).

DISCUSSION

A high degree of variability in the evaluation of mitogen-induced lymphoproliferative responses was observed in spite of special care in controlling for physiological (circadian rhythms, health, smoking, caffeine, medication, fasting), psychological (stress) and technical (technician, laboratory supplies, time and conditions of storage) factors likely to affect the assay, as suggested by Kiecolt-Glaser[18] and Fletcher *et al.*[14]

A time effect was observed for the overall dose–response curves of both PHA and ConA challenges. Significant differences were observed between T2

and T3, i.e. between the end of April and the middle of May. This period corresponds to spring in Quebec, when weather is extremely variable. Therefore the overall time effect could be attributed to a seasonal effect. A month effect for both PHA and ConA at this time of the year has also been recently observed by Van Rood *et al.*[19]

The time effect could also be attributed to day-to-day variation in performing the assay. In a recent study of depression and ConA-induced proliferative response, Darko *et al.*[20] stressed the high coefficient of variation of the assay within and between subjects associated with variation in the day of laboratory testing. To overcome this problem, he has suggested performing all assays on the same day by using cryopreservation techniques, or at least performing paired experimental and comparison subjects on the same assay day.

However, for the same homogeneous sample ($n = 10$), the blastogenic response induced by PHA used at 1:100 dilution and by ConA used at 5 μg/ml suboptimal concentration led to reasonably stable responses. Taking only the two first sampling days (T1 vs T2) and only looking at the first contrast, our findings are consistent with those of Boutet *et al.*,[12] who reported that sequential testing did not appear to affect PHA blastogenesis when the tests were performed twice in the same manner and at the same hour while controlling for age.

Unfortunately, most authors do not report test–retest coefficients. In our study, satisfactory G and G^* coefficients were found only for PHA and only at one dilution. For PHA, the G coefficient was borderline over two tests and more acceptable when measures were taken across three successive tests. It means that at least two series of measures are needed to obtain reliable results with PHA assay. These findings are consistent with Rodriguez (cited by Fletcher[14]). These authors obtained slightly better G coefficients (0.85), which could be explained by the use of a whole blood technique. They suggest that lymphocyte reactivity in whole blood may more accurately reflect their reactivity *in vivo.* Here we chose a lymphocyte separation procedure because most investigators in PNI used this procedure. For ConA, G and G^* coefficients in this study were only borderline for the 5 μg/ml concentration even when measures were taken across four successive tests. Unfortunately, no G coefficient or any other reliability coefficients have been published previously.

Greater variability in ConA could also mean that ConA lymphocyte stimulation is more sensitive to subtle changes than PHA. In two recent experimental studies,[10,11] laboratory stressor exposure decreased ConA-induced proliferation but did not produce significant change in PHA. In one of these studies,[10] the decreased effect was observed only at one ConA concentration (5 μg/ml). Future experimental studies should examine these important phenomena, namely, the reliability of baseline proliferation and reactivity to stressor-decreased effects, by looking at a wider range of mitogens and mitogen concentrations.

Experimental studies could also help to improve standardization and interpretability of the proliferative assays in PNI. Lymphocyte proliferation assay remains a non-specific *in vitro* assay. Mitogens stimulate a higher proportion of cell division than antigens usually do *in vivo*. This type of immune functional assay was first standardized for use in a clinical setting. Used as a clinical test, PHA or ConA lymphoproliferative response is sufficient to exclude severe cell-mediated immunodeficiencies. Used as a research tool in PNI, these assays require better standardization. Furthermore, ConA seems reasonably stable only at suboptimal dose. What does a suboptimal challenge response mean? According to Darko *et al.*[20] only severe and repeated depressions of proliferative response at optimal dose allow reasonable association with greater susceptibility to infection.

Until more experimental study results are available, caution is needed in interpreting changes in mitogen-induced lymphoproliferative responses. Some PNI authors have prematurely used proliferative response to formulate far-reaching data interpretation. The general lack of reliability found in this study emphasizes the need for caution in interpreting immune parameters. Our findings demonstrate the importance of having more than one dose of mitogen and the need to determine the optimum concentration for each lot and for each laboratory. Generalizability analysis indicated that at least two measurements for PHA and four for ConA can provide reliable measurement with careful control of several parameters.

However, we are aware of the fact that the assay reliability would benefit from being tested on a much larger sample size before a final judgement can be made regarding its usefulness as a tool in PNI studies.

SUMMARY

In summary, PHA-induced blastogenic assay demonstrated good test–retest reliability only at a 1:100 dilution. ConA-induced blastogenic assay demonstrated more instability and was acceptable only at the suboptimal dose of 5 μg/ml, which may lead to questions about the clinical meaning of this response. Results showed the importance of (1) selecting more than one concentration when using mitogen proliferation assay, (2) having at least two measurement times for PHA and four for ConA, and (3) controlling for circadian, day-to-day and seasonal variations when performing this kind of assay. Finally, it should be emphasized that new immunological measures need to be studied for their metric adaptability before transferring them from a clinical test to a PNI tool.

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REFERENCES

1. O'Leary, A. Stress, emotion and human immune function. *Psychol. Bull.* 1990; **108**: 363–382.
2. Biondi, M. and Pancheri, P. Mind and immunity. *Adv. Psychosom. Med.* 1987; **17**: 234–251.
3. Workman, E. A. and LaVia, M. F. Immunological effects of psychological stressors: A review of the literature. *Int. J. Psychosom.* 1987; **34**: 35–40.
4. Kiecolt-Glaser, J. K., Fisher, L. D., Ogrocki, P., Stout, J., Speicher, C. E. and Glaser, R. Marital quality, marital disruption, and immune function. *Psychosom. Med.* 1987; **49**: 13–32.
5. Halvorsen, R. and Vassend, O. Effects of examination stress on some cellular immunity functions. *J. Psychosom. Res.* 1987; **31**: 693–701.
6. Worman, E. A. and Lavia, M. F. T-Lymphocyte polyclonal proliferation: Effects of stress and stress response style on medical students taking national board examinations. *Clin. Immunol. Immunopathol.* 1987; **43**: 308–313.
7. Arnetz, B. B., Brenner, S., Levi, L., Hjelm, R., Petterson, I. L., Wasserman, J., Petrini, B., Kallner, A., Kvetnansky, R. and Vigas, M. Neuroendocrine and immunologic effects of unemployment and job insecurity. *Psychother. Psychosom.* 1991; **55**: 76–80.
8. Weisse, C. S. Depression and immunocompetence: A review of the literature. *Psychol. Bull.* 1992; **111**: 475–489.
9. Manuck, S. B., Cohen, S., Rabin, B. S., Buldoon, M. F. and Bachen, E. A. Individual differences in cellular immune response to stress. *Psychol. Sci.* 1991; **2**: 111–115.
10. Zakowzki, S. G., McAllister, C. G., Deal, M. and Baum A. Stress, reactivity, and immune function in healthy men. *Health Psychol.* 1991; **11**: 223–232.
11. Weisse, C. S., Pato, C. N., McAllister, C. G., Littman, R., Breier, A., Paul, S. M. and Baum, A. Differential effects of controllable and uncontrollable acute stress on lymphocyte proliferation and leukocyte percentages in humans. *Brain, Behav. Immun.* 1990; **4**: 339–351.
12. Boutet, R. A., Morse, P. A. and Watts, L. M. An analysis of PHA lymphoblastogenesis in whole blood. *Surg. Gynecol. Obstet.* 1978; **146**: 609–616.
13. Fitzgerald, M. A. The establishment of a normal human population dose–response curve for lymphocyte cultures with phytohemagglutinin. *Clin. Exp. Immunol.* 1971; **8**: 421.
14. Fletcher, M. A., Klimas, N., Morgan, R. and Gjerset, G. Lymphocyte proliferation. In: *Manual of Clinical Laboratory Immunology* (4th edn). Rose, N. E., Conway de Macario, E., Fahey, J. L., Friedman, H. and Penn, G. M. (Eds) American Society for Microbiology, Washington, DC, 1992, pp. 213–219.
15. Knapp, M. S. and Pownall, R. Lymphocytes are rhythmic: Is this important? *Brit. Med. J.* 1984; **289**: 1328–1330.
16. Lemyre, L., Tessier, R. and Fillion, L. *Mesure du Stress Psychologique (MSP): Manuel d'Utilisation. Behaviora*, Montréal, 1990.
17. Llabre, M. M., Ironson, G. H., Spitzer, S. B., Gellman, M. D., Weidler, D. J. and Schneiderman, N. How many blood pressure measurements are enough? An application of Generalizability Theory to the study of blood pressure reliability. *Psychophysiology* 1988; **25**: 97–106.
18. Kiecolt-Glaser, J. K. Methodological issues in behavioral immunology research with humans. *Brain Behav. Immun.* 1988; **2**: 67–78.
19. Von Rood, Y. R., Goulmy, E., Blokland, E., Pool, J., Vav Rood, J. and Van Houwelingen, H. Month related variability in immunological test results: Implications for immunological follow-up studies. *Clin. Exp. Immunol.* 1991; **86**: 349–354
20. Darko, D. F., Wilson, N. W., Gillin, J. C. and Golshan, S. A critical appraisal of mitogen-induced lymphocyte proliferation in depressed patients. *Am. J. Psychiat.* 1991; **148**: 337–344.