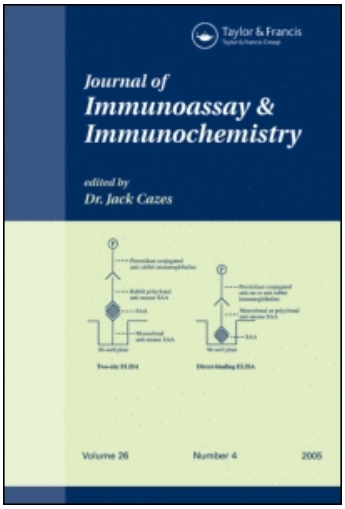

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Development of a Storage-Compatible Microtiter Plate-Based Technique for Lymphocyte Proliferation

R. P. Kerr[ab]; S. Kim[ab]; S. Ansar Ahmed[ab]; Steven D. Holladay[b]; R. M. Gogal Jr.[ab]
[a] The Center for Molecular Medicine and Infectious Diseases, Virginia Tech, Blacksburg, VA, USA [b] Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, USA

To cite this Article Kerr, R. P. , Kim, S. , Ahmed, S. Ansar , Holladay, Steven D. and Gogal Jr., R. M.(2008) 'Development of a Storage-Compatible Microtiter Plate-Based Technique for Lymphocyte Proliferation', Journal of Immunoassay and Immunochemistry, 29: 2, 128 — 142
To link to this Article: DOI: 10.1080/15321810801887680
URL: http://dx.doi.org/10.1080/15321810801887680

Journal of Immunoassay & Immunochemistry, 29: 128–142, 2008

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810801887680

Development of a Storage-Compatible Microtiter Plate-Based Technique for Lymphocyte Proliferation

R. P. Kerr,[1,2] S. Kim,[1,2] S. Ansar Ahmed,[1,2] Steven D. Holladay,[2] and R. M. Gogal, Jr. [1,2]

[1]The Center for Molecular Medicine and Infectious Diseases, Virginia Tech, Blacksburg, USA
[2]Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, USA

Abstract: The lymphocyte proliferation assay (LPA) is an important tier 1 test to assess non-specific lymphocyte function, *in vitro*. However, this assay requires fresh preparation, is time consuming, and labor intensive. Developing a plate coating technique for lymphocyte proliferation that is both stable and storage compatible would be useful to the basic and clinical researcher. In this study, we compared the effects of different mitogen plate coating techniques on lymphocyte proliferation to freshly prepared plates. The results show that plates prepared with complete media and stored at −40°C up to 10 days corresponded well to control plates.

Keywords: Lymphocyte, Proliferation, Mouse, Mitogen, Plate-coating, *In vitro*

INTRODUCTION

Lymphocyte proliferation assays (LPA), also referred to as blastogenesis or lymphocyte transformation assays, are routinely used to assess the function and viability of lymphocytes, *in vitro*.[1] The degree of cell growth or proliferation corresponds with the type and concentration of mitogen as well as the duration of stimulus. Clinically, mitogen-induced proliferation analysis can provide some insight into an individual's immunocompetency.[2] For

Correspondence: R. M. Gogal, Jr, The Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia Tech, 1410 Prices Fork Rd., Blacksburg, VA 24060-0342, USA. E-mail: rgogal@vt.edu

example, lymphocyte proliferation assays can be utilized in clinical immunology laboratories to help assess both T-cell and B-cell function in patients suspected of having some form of immunodeficiency.[1,3]

Common mitogens employed in LPAs include concanavalin A (Con A) and lipopolysaccharide (LPS).[1,4] Con A, a plant lectin obtained from the jack bean (*Canavalia ensiformis*), is a recognized Ca^{2+}-dependant T-cell stimulant in mammals.[1,5,6] Bacterial lipopolysaccharides (LPS) are an important component of the outer membrane of Gram-negative bacteria. LPS consists of three regions: lipid A, core oligosaccharides, and polysaccharide side chains.[7] LPS has been shown to be an effective polyclonal activator of B-lymphocytes and macrophages.[8]

Lymphocyte proliferation can be delivered by various proliferation marker assays. These include: the [^{3}H] thymidine incorporation assay, where radioactive [^{3}H] thymidine is incorporated into DNA, is the most commonly used assay.[1,9] Other assays include the use of the tetrazolium salts; MTT (3-(4,5-di-methyethiazol-2-yl)-2,5-diphenyltetrazolium bromide), and XTT (sodium 3′-[1-phenylamino)-carbonyl]-3,4-tetrazolium-*bis*(4-methoxy-6-nitro)benzene-sulfonic acid hydrate).[1] The MTT colorimetric assay uses the metabolism of the yellow tetrazolium salt in viable cells into blue crystals as the detection agent. XTT is similar to MTT, except the tetrazolium salt metabolizes into orange crystals.[10] The BrdU-assay and the alamarBlue™ Assay are new alternative methods that are also used to assess lymphocyte proliferation. The BrdU assay (5-bromodeoxyuridine) is a pyrimidine analogue that is incorporated into DNA instead of using [^{3}H]thymidine.[10] The alamarBlue™ assay utilizes an oxidative-reduction dye that has colorimetric and fluorometric properties and does not appear to damage cells. The dye changes color from blue (the oxidized form) to red (the reduced form) when cells undergo metabolic changes such as proliferation.[1,11,12]

The measurement of lymphocyte proliferation is a commonly employed assay in immunology-based research.[1] However, plate preparation can be both time consuming and labor intensive.[3] Further, the preparation of these plates is commonly performed on the day of study (i.e., fresh preparation). This requirement effectively reduces workflow volume, as time that could have been used for additional sample analysis must be reserved for the preparation of the assay plates. Additionally, the fresh preparation method may result in inter-assay variation. Thus, the creation of a standardized mitogen plate-coating protocol resulting in the ability to generate an LPA plate with long-term stability would be of use to the researcher conducting either proliferation or cytokine studies. The potential benefits could include decreased hands-on time for plate preparation on the day of study, as well as better inter-assay results across multiple experimental time points.

In this study, we explored various plate coating media in the quest to develop a stable proliferation plate that compared well to freshly prepared mitogen plates. The coating vehicles used were acetone, ethanol, PBS, media supplements, and complete media as solvents in various

volumes. Storage temperature and post-dispense coatings, including evaporation and lyophilization, were also evaluated. Two concentrations of Con A and LPS were used to evaluate the influence of plate coating techniques on T and B-cell function, respectively. Alamarblue™ dye was employed to track the effectiveness of the plate-coating techniques.

EXPERIMENTAL

Mice

Eleven week-old female C57Bl/6 mice were maintained at the Non-Client Animal Facility at the Center for Molecular Medicine and Infectious Diseases (Blacksburg, VA). Mice were fed a commercial pelleted diet and provided water *ad libitum*. All animals were housed at 22°C, humidity of 40–60%, and a 14/10 hr light/dark cycle.[13,14] All experimental animals were cared for and maintained in accordance with the VPI & SU Animal Care Committee (ACC) guidelines.

Experimental Design

Six separate experiments were performed in this study to evaluate several coating media and volumes, and storage durations. For each experiment, a freshly prepared proliferation plate was used as control. Specific absorbance values from these plates were also used to analyze inter-assay variation. In each experiment, the spleens of 4 mice were utilized for evaluating spontaneous and mitogen-induced proliferation.

Cell Culture Plate Preparations

Complete Media

Corning 96-well tissue culture plates (Corning, Corning, NY) were prepared just prior to culture or stored for 10 days at −40°C as noted. Complete medium was prepared by combining RPMI-1640, 1% penicillin/streptomycin, 1% non-essential amino acids, 25 mM HEPES buffer (MediaTech, Hendon, VA), and 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, Atlanta, GA). One hundred microliter aliquots of complete media containing 1 μg/well Con A, 2.5 μg/well Con A, 1 μg/well LPS, or 2.5 μg/well LPS (Sigma, St. Louis, MO) were dispensed in triplicate and immediately frozen for storage or placed in culture after dispensing of cell suspensions (Table 1).

Table 1. Preparation and storage methods evaluated

Preparation method	Storage time	Storage method		
		4° Refrigerator	−40° Freezer	Lyophilized
100 μl Complete media (Fresh)	0 hr	X	—	—
10 μl Ethanol, evaporated	48 hr	X	X	—
20 μl Acetone, evaporated	48 hr	X	X	—
10 μl Ethanol, no evaporation	48 hr	X	X	—
20 μl Acetone, no evaporation	48 hr	X	X	—
20 μl PBS	96 hr	—	X	X
30 μl Media supplements	96 hr	—	X	X
20 μl PBS	10 d	—	X	X
30 μl Media supplements	10 d	—	X	X
20 μl Complete media	24 hr	—	X	—
20 μl Complete media	10 d	—	X	—
100 μl Complete media	10 d	—	X	—

Low Volume Complete Media

Corning 96-well tissue culture plates were prepared and stored for 1 or 10 days at −40°C as noted. Twenty microliter aliquots of complete media containing 1 μg/well Con A, 2.5 μg/well Con A, 1 μg/well LPS, or 2.5 μg/well LPS were dispensed in triplicate and immediately frozen (Table 1).

Ethanol or Acetone

Corning 96-well tissue culture plates were prepared and stored for 48 hr. Ten microliter aliquots of sterile ethanol (Sigma, St. Louis, MO) containing 1 μg/well Con A, 2.5 μg/well Con A, 1 μg/well LPS, or 2.5 μg/well LPS were dispensed in triplicate and immediately stored at −40 or 4°C, or allowed to evaporate for 25 min at room temperature and then stored at −40 or 4°C as noted. Acetone tissue culture plates were prepared similar to ethanol using 20 μL aliquots of sterile acetone (Sigma, St. Louis, MO) (Table 1).

Phosphate Buffered Saline

Cold phosphate buffered saline (PBS) (Mediatech, Herndon, VA) was used as vehicle to dilute Con A and LPS. Twenty microliter aliquots containing 1 μg/well Con A, 2.5 μg/well Con A, 1 μg/well LPS, or 2.5 μg/well LPS were dispensed in triplicate and immediately frozen (Table 1).

Media Supplements

Sixty percent FBS, 13.3% non-essential amino acids, 13.3% penicillin/streptomycin and 20% PBS were combined into a concentrated media supplement that was dispensed into 96-well tissue culture plates. Thirty microliters of ether vehicle alone, 1 μg/well Con A, 2.5 μg/well Con A, 1 μg/well LPS, or 2.5 μg/well LPS were dispensed in triplicate and immediately frozen. Plates were then stored, or lyophilized and stored for 4 or 10 days at −40°C as noted (Table 1).

Splenic Isolation

Splenic lymphocytes were isolated under sterile conditions by a procedure described previously by Gogal et al.[1,15] Briefly, splenic tissues were gently pressed against a metallic sieve screen (Sigma, St. Louis, MO) until cells were suspended in RPMI media (Mediatech, Herndon, VA). Once dissociations were complete, cell suspensions were centrifuged (10 min, 250 × g and 7°C) and supernatants discarded.[14] Cells were then resuspended in one mL RPMI incomplete media. To each tube, 3 mL ACK lysis buffer, pH 7.2, were added, for 5 min.[14] Cells were then washed 2× with RPMI incomplete media, centrifuged (10 min, 250 × g and 7°C), supernatant discarded, and concentrations adjusted in RPMI media. Cells were enumerated on a Beckman Coulter Multisizer 3 particle counter (Beckman-Coulter, Fullerton, CA).

Lymphocyte Proliferation Assays

Enriched splenic lymphocyte suspensions were dispensed into 96-well tissue culture plates at 5×10^5 cells/200 μL/well. After 48 hr culture, 20 μL Alamarblue™ metabolic dye was added to each well. After 72 hr of culture, the plates were read on a Spectramax plate reader running Softmax version 3.2 (Molecular Devices, Sunnyvale, CA). Results were expressed as Δ specific absorbance (570 nm−600 nm) calculated by subtracting the mean media and cell only values from the media, cell, and mitogen values for each sample.

Statistics

The results were tabulated in Microsoft Excel (Microsoft, Redmond, WA). A standardized protocol to reduce experimental error was implemented to remove outlier data. When intra-well standard deviation for the same sample and mitogen concentration exceeded 15% of the mean, wells which

differed greater than 1.96 standard deviations from the mean were removed and means and standard deviations recalculated. If the recalculated standard deviation was greater than 15% of the recalculated mean, that sample was excluded from analysis. Remaining data points were then analyzed in SPSS using the two-tailed K-independent samples' test, two-tailed student's t-tests and single-tailed Pearson's Coefficient analysis where applicable (SPSS Inc., Chicago, IL). Results were considered statistically significant at the $p \leq 0.05$ level with $n \geq 3$ samples/coating medium/mitogen/concentration.

RESULTS AND DISCUSSION

Mice

The mean mouse body weight of the mice used for all six of the experiments was 35.93 ± 2.74 grams. The mean splenic weight and mean splenic cellularity were 92.0 ± 5.0 mg and $54.02 \pm 4.10 \times 10^6$ cells, respectively. There were no significant differences across body weight, splenic weight, cellularity, or age of the mice. All animals were continuously monitored daily and were in good health throughout the study.

Assessment of Spontaneous Proliferation

The quantification of spontaneous cell proliferation cultured in media is a useful marker to assess inter-assay variation. It provides the investigator with an internal control to verify the viability, dilution and pipette accuracy of cell suspensions. Mean specific absorbance values of media- and cell-only wells for all six experiments were similar regardless of coating medium, temperature, timeframe, or storage method, except for lyophilized media supplement plates (data not shown). Cells cultured in media supplement-coated plates that were lyophilized failed to support cell growth, suggesting that lyophilization degraded the media supplements, making them unsuitable to sustain cell growth.

Ethanol and Acetone; Evaporated

In this experiment, Con A and LPS were dissolved in ether ethanol or acetone aliquoted into wells of a Corning 96-well tissue culture plate, subjected to 25 min evaporation at room temperature, then stored for 48 hr at either 4°C or −40°C (Figure 1). Con A, dissolved in either ethanol or acetone, resulted in significantly reduced proliferation compared to the fresh control plate. LPS dissolved in ethanol, at 1.0 μg/well, showed a decreasing mean Δ specific absorbance non-significant trend. This decline in mean Δ specific

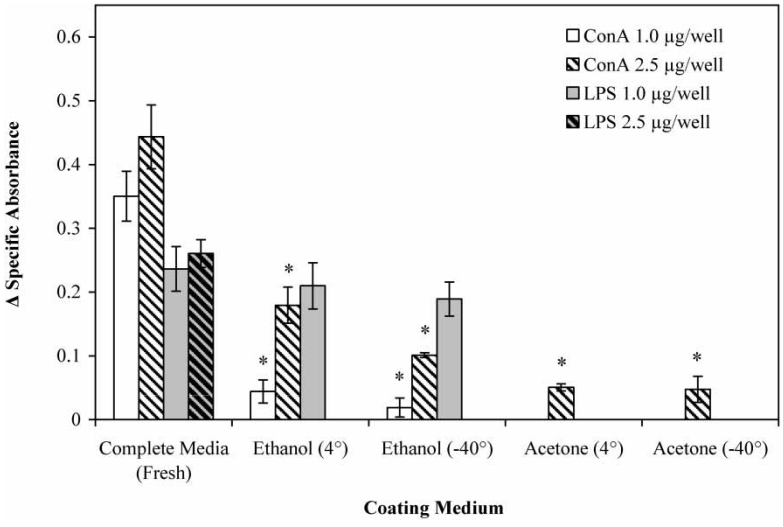

Figure 1. Two 96-well culture plates containing Con A and LPS dissolved in ethanol and aliquoted at 10 μL/well were allowed to evaporate for 25 min. One plate was stored at 4°C and the other at −40°C for 48 hr. Two additional plates with mitogens dissolved in acetone (20 μL/well) were similarly prepared. A fresh plate prepared on the day of study with mitogens diluted in 100 μL complete media/well served as control. AlamarBlue™ was added at 48 hr culture and read at 72 hr. Results are expressed as Δ specific absorbance ± SEM. ($^*p \leq 0.05$, Students *t*-test, $n \geq 3$ *mice/coating medium*).

absorbance with decreasing storage temperature may be a result of decreased evaporation of ethanol. It would have been interesting to see if the higher LPS dissolved in ethanol would have shown the same trend. Unfortunately, low splenic cellular recoveries precluded testing LPS at 2.5 μg/well. Mitogens dissolved in acetone and plated into 96-well tissue culture plates, regardless of concentration, failed to stimulate the cultured splenocytes of the LPS-acetone coated wells and showed a barely detectable Δ specific absorbance with the Con A at 2.5 μg/well as such were significantly different from control.

Ethanol and Acetone; Non-Evaporated

We next evaluated lymphocyte proliferation with mitogens dissolved in either ethanol or acetone, pre-coated in 96-well tissue culture plates, then stored at either 4°C or −40°C (Figure 2). Thus, these series of plates were prepared similarly to the first experiment but without an evaporative step. Compared to the control, plates pre-coated with Con A dissolved in either ethanol or acetone yielded significantly decreased Δ specific absorbencies, irrespective

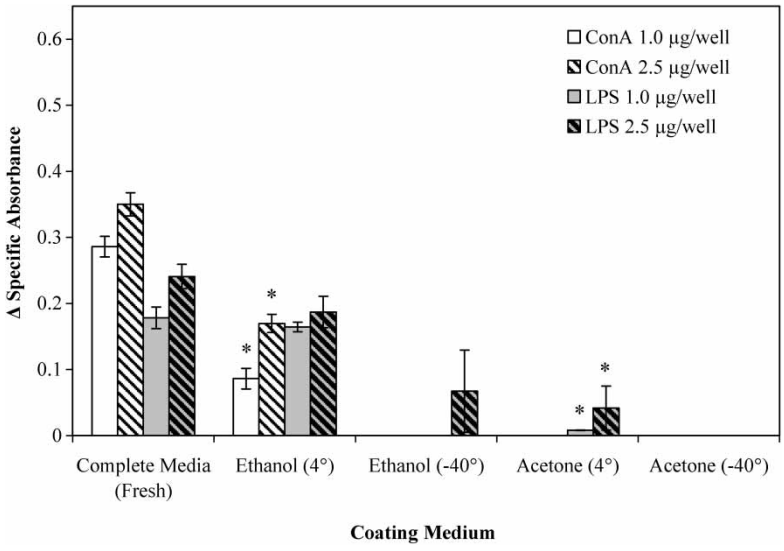

Figure 2. Two 96-well culture plates containing Con and LPS, dissolved in ethanol and aliquoted at 10 μL/well, were immediately stored at 4°C and the other at −40°C for 48 hr. Two additional plates with the mitogens dissolved in acetone (20 μL/well) were similarly prepared. A fresh plate prepared on the day of study with mitogens diluted in 100 μL complete media/well served as control. AlamarBlue™ was added at 48 hr culture and read at 72 hr. Results are expressed as Δ specific absorbance ± SEM. (*$p \leq 0.05$, Students *t*-test, $n \geq 3$ *mice/coating medium*).

of storage temperature. As observed with the pre-coated plates in the previous experiment, mitogens dissolved in acetone and pre-coated in wells generated mean Δ specific absorbencies well below 0.1. Values that are below a 0.1 Δ specific absorbance denote a lack of stimulatory capacity.[1] Similar to the previous experiment involving ethanol as diluent and coating agent, the pre-coated plates containing LPS dissolved in ethanol showed a non-significant decreasing trend in mean Δ specific absorbance. This lack of significance, however, is not supported by regression ($r = -0.821$, $p = 0.066$) or plate contour map analysis. Plate contour mapping demonstrated a clear evaporation pattern, with wells on the edge of the plate having higher specific absorbencies than wells in the interior. This contributed to the higher variability and subsequently lower specific absorbance values observed on the −40°C ethanol coated plate.

PBS and Media Supplements: Evaluation of Lyophilization

We next examined the efficacy of PBS and combined media supplements as plate coating vehicles. The use of neutral pH buffered mediums (e.g., PBS)

is commonly employed to dilute and store stock solutions of Con A and LPS. The rationale for evaluating media supplements is that they are essential components added to incomplete medium (e.g., RPMI-1640) to generate a complete medium capable of promoting cell proliferation. Thus, if media supplements could be utilized as pre-coating vehicle whilst still maintaining Con A and LPS mitogenic potency, cells could be resuspended in an incomplete medium and pipetted directly into the wells. This would also facilitate the use of extra cells in other analyses (e.g., cytology, flow cytometry). Additional to the different coating media tested, extra plates containing mitogens coated with PBS or media supplements were subjected to lyophilization and evaluated for proliferation response.

All of the plates were stored at −40°C and evaluated after 4 days or 10 days. The proliferation indices for the PBS-coated plates, lyophilized PBS-coated plates, and the media supplement-coated plates after 4 days of storage at −40°C displayed a proliferation profile for Con A and LPS similar to control but with lower Δ specific absorbencies that was statistically different from control (Figure 3). Despite these differences, LPS dissolved in PBS Δ specific absorbencies at both 1.0 μg/well ($r = 0.986$, $p = 0.007$) and 2.5 μg/well ($r = 0.997$, $p = 0.002$) stored for 4 days correlated significantly and

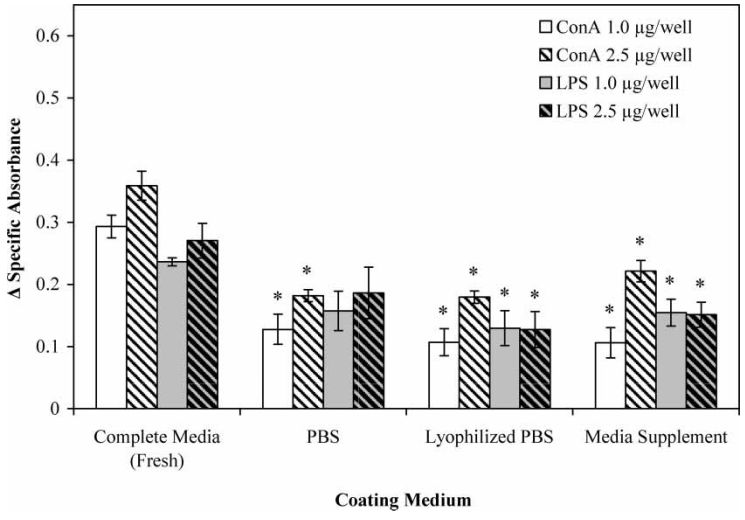

Figure 3. Con A and LPS were resuspended in cold PBS and in 30 μL/well aliquots, dispensed into two 96 well culture plates then stored at −40°C or lyophilized and stored at −40°C for 4 days. Two additional plates with the mitogens dissolved in concentrated media supplements (30 μL/well) were similarly prepared. A fresh plate prepared on the day of study with mitogens diluted in complete media and dispensed in 100 μL/well aliquots served as control. AlamarBlue™ was added at 48 hr culture and read at 72 hr. Results are expressed as Δ specific absorbance ± SEM. (*$p \leq 0.05$, Students *t*-test, $n \geq 3$ *mice/coating medium*).

strongly with control plates. Neither Con A concentrations correlated with control. Interestingly, lyophilized media supplement-coated plates did not sustain viable cell cultures (data not shown). Lyophilization is a process that takes a frozen material and dehydrates it under low pressure. This would appear to suggest that lyophilization of these plates degraded the media supplements to a point of making them unable to support cell growth. Evaluation of the proliferation indices of plates stored for 10 days at −40°C showed some similarities and some variations in proliferation indices in coated plates compared to the plates stored for 4 days at −40°C. Wells containing Con A that was dissolved in either PBS or media supplements showed a similar proliferation pattern to the two Con A concentrations, but with much lower Δ specific absorbencies than controls such that they were statistically different (Figure 4). The same statistically different Δ specific absorbencies were seen in the lyophilized PBS coated plates stored for 10 days. However, plates stored for 10 days containing wells with LPS dissolved in PBS, LPS dissolved in PBS then lyophilized, and LPS dissolved in media supplements were comparable to control proliferation indices.

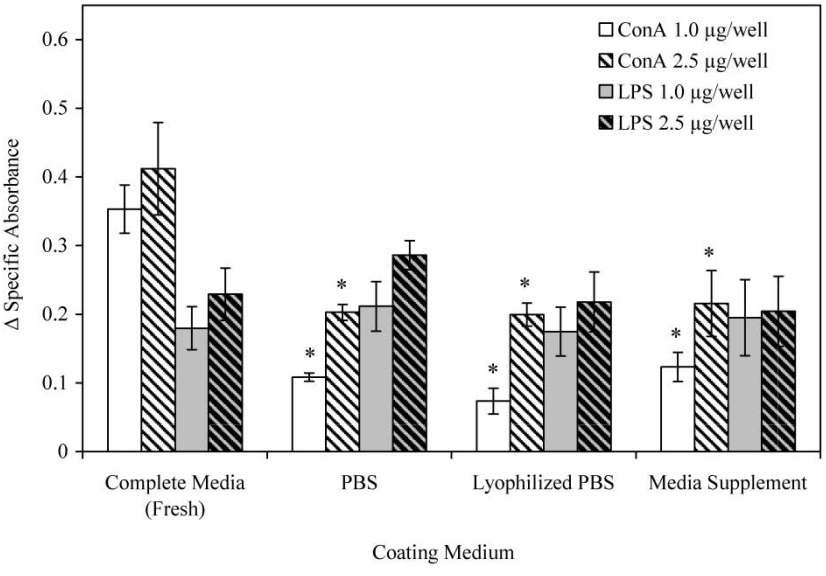

Figure 4. Con A and LPS were resuspended in cold PBS and in 30 µL/well aliquots, dispensed into two 96 well culture plates then stored at −40°C or lyophilized and stored at −40°C for 10 days. Two additional plates with the mitogens dissolved in concentrated media supplements (30 µL/well) were similarly prepared. A fresh plate prepared on the day of study with mitogens diluted in complete media and dispensed in 100 µL/well aliquots served as control. AlamarBlue™ was added at 48 hr culture and read at 72 hr. Results are expressed as Δ specific absorbance ± SEM. (*$p \leq 0.05$, Students *t*-test, $n \geq 3$ *mice/coating medium*).

Complete Media

The results from the previous diluent pre-coating study appeared to indicate that vehicle diluents containing a neutral pH generated Δ specific absorbencies that were more comparable to control plates. Our final study focused on the use of complete media as a diluent and pre-coating vehicle for Con A and LPS. Preliminary studies in our laboratory, showed that freshly prepared plates containing Con A and LPS in complete media at 100 μL aliquots could be frozen −40°C for 24 hr and generate Δ specific absorbencies strongly comparable to freshly prepared plates (unpublished data). Thus, we decided to conduct a more thorough experiment involving different coating volumes (20 μL and 100 μL) of mitogen in complete media and a longer storage period (10 day). In the previous experiments, a 20 μL volume of the mitogen in vehicle diluent was evaluated. To compare the effectiveness of complete media as a vehicle, Con A and LPS were diluted in complete media and dispensed at 20 μL/well and plates frozen at −40°C for 24 hr. The Δ specific absorbencies of the plate stored at −40°C for 24 hr were non-significantly different from freshly prepared control plates for each mitogen at each concentration (Figure 5). A significant strong correlation

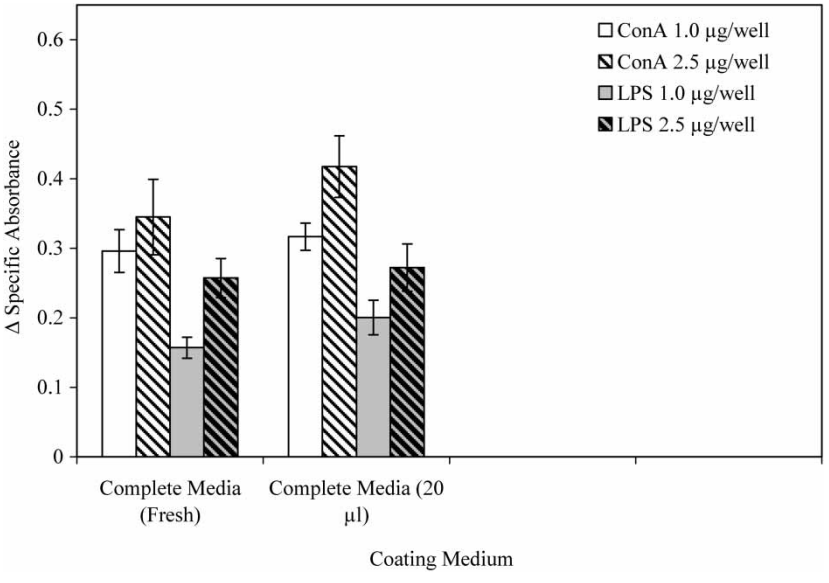

Figure 5. Complete medium was used to dilute Con A and LPS. One 96-well culture plate received 20 μL of concentrated mitogens and then stored at −40°C for 24 hr. A fresh plate prepared on the day of study with mitogens diluted in complete media and dispensed in 100 μL/well aliquots served as control. AlamarBlue was added at 48 hr culture and read at 72 hr. Results are expressed as Δ specific absorbance ± SEM. (*$p \leq 0.05$, Students *t*-test, $n \geq 3$ *mice/coating medium*).

between the 20 μL complete media pre coated plate containing Con A 1.0 μL/well and control ($r = 0.996$, $p = 0.028$), Con A 2.5 μg/well and control ($r = 0.969$, $p = 0.015$), and a strong, non-significant correlation between LPS 2.5 μg/well and control ($r = 0.940$, $p = 0.104$) were present. Low volume complete media (20 μL) coated LPS 1.0 μg/well correlated well with control but neither strongly nor significantly ($r = 0.741$, $p = 0.188$). Based on these results, we repeated the study with plates pre-coated with mitogens at 20 μL or 100 μL volumes of complete media that were stored at −40°C for 10 days. The Δ specific absorbencies of the mitogen response for the 20 μL or 100 μL complete media pre-coated plates were not significantly different from freshly prepared control plates regardless of mitogen or concentration in the k independent samples test (Figure 6). Low and nominal volume complete media coated mean Δ specific absorbencies were strongly correlated to control for each mitogen at each concentration. However only three of these correlations were significant; nominal volume Con A 2.5 μg/well ($r = 0.972$, $p = 0.013$), nominal volume LPS 1.0 μg/well ($r = 0.997$, $p = 0.001$), and low volume LPS

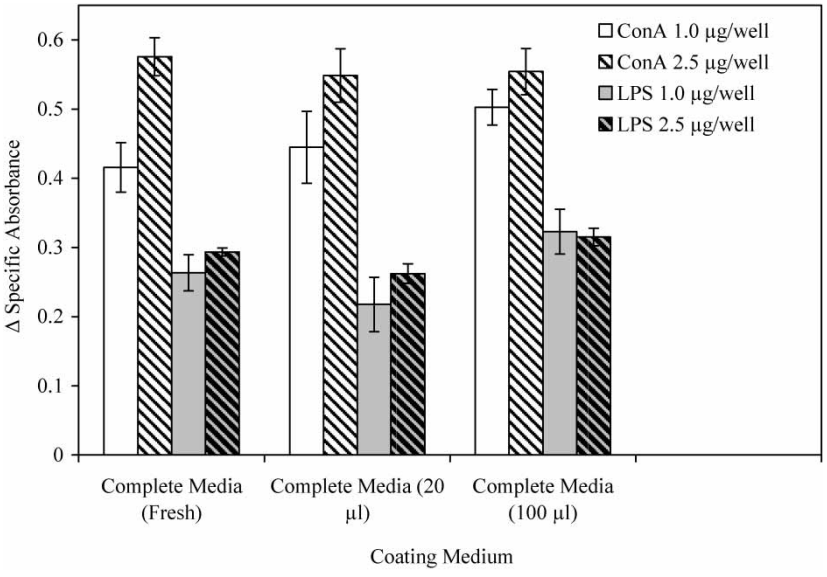

Figure 6. Complete medium was used to dilute Con A and LPS to two working concentrations to allow for different delivery volumes. One 96-well culture plate received 100 μL aliquots of the mitogens and the other plate received 20 μL of concentrated mitogens and the plates were stored at −40°C for 10 days. A fresh plate prepared on the day of study with mitogens diluted in complete media and dispensed in 100 μL/well aliquots served as control. AlamarBlue was added at 48 hr culture and read at 72 hr. Results are expressed as Δ specific absorbance ± SEM. ($^*p \leq 0.05$, Students *t*-test, $n \geq 3$ *mice/coating medium*).

1.0 μg/well ($r = 0.992, p = 0.039$). Additionally, four mean Δ specific absorbencies were strongly but slightly non-significantly correlated: Nominal volume Con A 1.0 μg/well ($r = 0.962$, $p = .085$), low volume Con A 1.0 μg/well ($r = 0.963$, $p = 0.084$), low volume Con A 2.5 μg/well ($r = 0.945$, $p = 0.100$), and low volume LPS 2.5 μg/well ($r = 0.867$, $p = 0.147$). The nominal volume LPS 2.5 μg/well group did not correlate with control at all ($r = 0.008, p = 0.146$) but the mean Δ specific absorbencies are so close that the mean ΔΔ standard absorbance (0.0216) was actually smaller than the standard deviation (0.0258), indicating that the means are so close that the experimental error is greater than the difference between the groups. This lack of statistical difference between means, when taken with the strength of the correlations between novel and routine plate coating mediums, clearly demonstrates the feasibility of using frozen plates (for up to 10 days) containing mitogens dissolved in complete media and coated at either 20 μL or 100 μL volumes to perform proliferation assays.

CONCLUSIONS

In large sample studies, a considerable amount of experiment time is necessary for proper preparation of the plates. Unfortunately, this could limit a researcher's ability to collect and analyze other sample endpoints during the day of the experiment due to time constraints. An additional concern associated with generating fresh plates is the increased opportunity for inter-assay variation. Variations in proliferation data between experiments conducted at different time points can weaken the statistical analysis of the data. Therefore, developing a proliferation plate that can be prepared in large quantities, has a stable shelf life, and correlates strongly with freshly prepared plates with minimal inter-assay variation would be useful to both the basic science and clinical researcher. To begin to address this goal, we examined a number of different vehicle diluents, a variety of temperature storage conditions, a range of coating volumes, as well as lyophilization.

In the first sets of experiments, we examined if two low boiling point solvents (acetone and ethanol) could be employed as mitogen coating diluents. Due to the low boiling point of these solvents and high melting point of the mitogen, we hypothesized that the solvent would evaporate away leaving behind the mitogen in the well. Although ethanol was effective for LPS, it was unsuitable for Con A. Acetone was ineffective in both respects. When there is no allowance for evaporation, results are effected dramatically. The toxicity of these organic solvents appears to negatively affect the plated mitogen. As Con A is sensitive to changes in pH, there is a possibility that the solvent could have caused conformational changes in the molecule. At physiological pH, Con A exists as a tetramer and is stable in culture media (incomplete RPMI media, pH of 7.3).[16] Under acidic conditions (pH ~5.0) however, Con A undergoes a conformational change and

converts to a homodimer configuration.[17] RPMI incomplete media has a pH of 7.3. The acetone and ethanol used in this study had pHs of 6.7 and 6.1, respectively. These slightly acidic conditions may have had an effect on Con A in solution. Interestingly, LPS was much more stable than Con A in ethanol.

For the second series of experiments, PBS and media supplements were selected as coating mediums because they are non-toxic and have longer shelf lives when stored properly. Again we saw that Con A was less stable than LPS and that PBS served as a good coating medium for LPS out to 4 days. Wells coated with media supplements did not perform as expected and failed to support proliferation after lyophilization. Consequently, LPS appeared again to be more stable than Con A, but using low volume PBS, LPS only assays may be prepared up to 4 days ahead of time.

Although we had limited success in finding coating agents for LPS, none of the pervious coating mediums supported Con A mitogenicity. Concern over the potential pH sensitivity of Con A mandated a switch in the focus back to using complete media (RPMI-1640 + media supplements) as a coating media. Plates prepared and then frozen at -40°C performed comparably to freshly prepared plates at both low and nominal volumes, with both Con A and LPS at both concentrations. We conclude that using complete media as a coating agent for mitogens is suitable for pre-preparation of lymphocyte proliferation plates up to 10 days ahead of time and potentially longer. Future studies are recommended to examine longer storage times and other coating volumes.

ACKNOWLEDGMENT

The authors would like to thank Ms. Shannon Viers for the care and maintenance of the animal colonies.

REFERENCES

1. Ahmed, S.A.; Gogal, R.M., Jr.; Walsh, J.E. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H] thymidine incorporation assay. J. Immunol. Meth. **1994**, *170* (2), 211–224.
2. Pennebaker, J.W.; Kiecolt-Glaser, J.K.; Glaser, R. Discloser of traumas and immune function: health implications for psychotherapy. J. Consult. Clin. Psychol. **1988**, *56* (2), 239–245.
3. Weinberg, A.; Betensky, R.A.; Zhang, L.; Ray, G. Effect of shipment, storage, anticoagulant, and cell separation on lymphocyte proliferation assays for human immunodeficiency virus-infected patients. Clin. Diag. Lab. Immunol. **1998**, *5* (6), 804–807.

4. Gogal, R.M.; Ahmed, S.A.; Larsen, C.T. Analysis of avian lymphocyte proliferation by a new, simple, non-radioactive assay (Lympho-pro). Avian Dis. **1997**, *41* (3), 714–725.
5. Agrawal, B.B.L.; Goldstein, I.J. Physical and chemical characterization of concanavalin A, the hemagglutinin from jack bean (*Canavalia ensiformis*). Biochim. Biophys. Acta-Protein Struct. **1967**, *133* (2), 376–379.
6. Drickamer, K. Two distinct class of carbohydrate-recognition domains in animal lectins. J. Biol. Chem. **1988**, *263* (20), 9557–9560.
7. Raetz, C.R.H.; Whitfield, C. Lipopolysaccharide endotoxins. Ann. Rev. Biochem. **2002**, *71*, 635–700.
8. Sultzer, B.M. Genetic analysis of lymphocyte activation by lipolysaccharide endotoxin. Infect. Immun. **1976**, *13* (6), 1579–1584.
9. Meydani, S.N.; Ha, W.K. Immunological effects of yogurt. Amer. J. Clin. Nutr. **2000**, *71* (4), 861–872.
10. Wagner, U.; Burkhardt, E.; Failing, K. Evaluation of canine lymphocyte proliferation: comparison of three different colorimetric methods with the [^{3}H]-thymidine incorporation assay. Vet. Immunol. Immunopathol. **1999**, *70* (3–4), 151–159.
11. Nakayama, G.; Caton, M.; Nova, M.; Parandoosh, Z. Assessment of the Alamar Blue assay for cellular growth and viability in vitro. J. Immunol. Meth. **1997**, *204* (2), 205–208.
12. O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. **2000**, *267* (17), 5421–5426.
13. Gogal, R.M.; Prater, M.R.; Smith, B.J.; Johnson, M.S.; Holladay, S.D. Bilateral dissected spleens and thymuses in rodents exhibit homogeneity in leukocyte markers. Toxicology **2001**, *157* (3), 217–223.
14. Klein, A.B.; Witonsky, S.G.; Ahmed, S.A.; Holladay, S.D.; Gogal, R.M.; Link, L.; Reilly, C.M. Impact of different cell isolation techniques on lymphocyte viability and function. J. Immunoassay Immunochem. **2006**, *27* (1), 61–76.
15. Gogal, R.M.; Ahmed, S.A.; Holladay, S.D.; Walsh, J.E.; Galaburda, A.M.; Rosen, G.D. Induced minor malformations in the neocortex of normal mice do not alter immunological functions. Immunol. Invest. **2000**, *29* (3), 299–318.
16. Solis, D.; Estremera, D.; Usobiaga, P.; Diaz-Maurino, T. Differential binding of mannose-specific lectins to the carbohydrate chains of fibrinogen domains D and E. Eur. J. Biochem. **1987**, *165*, 131–138.
17. Wang, J.; Edelman, G. Binding and functional properties of concanavalin A and its derivatives. J. Biol. Chem. **1978**, *253* (9), 3000–3007.

Received November 4, 2007
Accepted November 13, 2007
Manuscript 3260