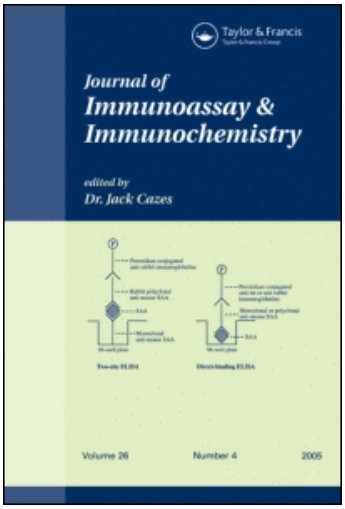


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# Viability and Function in Lymphocytes Cultured from the Horse, Chicken, and Mouse: Effects of Different Leukocyte Enrichment Techniques

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# Viability and Function in Lymphocytes Cultured from the Horse, Chicken, and Mouse: Effects of Different Leukocyte Enrichment Techniques

**A. Mustafa,[1] L. Gillmeister,[1] W. P. Hernandez,[4] C. T. Larsen,[2] S. Witonsky,[2] S. D. Holladay,[1] R. P. Kerr,[1] S. A. Ahmed,[1] A. Santo,[3] and R. M. Gogal, Jr[1]**

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**Abstract:** Methods of lymphocyte enrichment tend to vary across species, with the most common techniques employed being density-gradient separation and erythrocyte lysis buffer enrichment. In this study, we assessed lymphocyte viability and proliferation of avian, equine, and murine lymphocytes enriched by a commercial density-gradient technique and under identical, standardized culture conditions. The results of this study clearly show that, under identical enrichment and culture conditions, lymphocyte viability and function can be quite different among the equine, bird, and mouse species. Secondly, the type of enrichment technique employed in the mouse can impact the quality of the immune data generated.

**Keywords:** 7-AAD, Chicken, Horse, Lymphocyte isolation, Mouse, Viability

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## INTRODUCTION

We recently explored the influence of different dissociation techniques on lymphocyte viability, recovery, and proliferation.[1] A noteworthy observation was that lymphocyte proliferation varied significantly by type of tissue dissociation technique employed. This implied that investigators who study immunologic endpoints in toxicity or other studies might obtain differing results based on dissociation technique.

The present study similarly examined two techniques commonly utilized in lymphocyte enrichment, i.e., density-gradient centrifugation and erythrocyte (RBC) lysis buffer,[2,3] for effects on lymphocyte viability and function.

Of the species used in immunology-based studies, the rodent is the most common, as well as most relevant to humans.[4] The rodent spleen is the secondary lymphoid organ commonly used for the enrichment of mature lymphocytes.[4,5] Trypan blue exclusion and flow cytometric analysis of lymphocyte subsets show that T cells collected from the murine spleen are comparable in both cell marker expression and viability to murine cells collected from peripheral blood (Gogal and Hernandez, results unpublished). Therefore, the use of the mouse spleen as a source of mature lymphocytes is efficacious when desired lymphocyte yields from the peripheral blood are not achievable.



The utilization of cell membrane lysis buffers is common, following tissue dissociation, to remove red blood cells from lymphocyte suspensions. This technique is frequently used to enrich splenic lymphocytes in rodents.[1] Density gradient techniques are also widely used for leukocyte enrichment from blood or spleen. The gradient Lymphoprep™ (specific gravity 1.077) is used to separate a mixed population of cells into two major groups, a top fraction of low-density cells (mononuclear cells) and bottom fraction with cells of higher density.[6] Following centrifugation, the mononuclear cells (monocytes and lymphocytes) are located on the top of the separation medium, whereas erythrocytes and granulocytes sediment to the bottom. Cells of interest can be isolated either by positive selection or by removal of unwanted cells.

Previous studies involving different animal models in our laboratory suggested that equine, avian, and murine lymphocytes differ in both viability and proliferative capacity, following optimization of the lymphocyte enrichment procedures for each species.[7–9] These observations were limited, however, in that individual species were being evaluated, and a simultaneous analysis of the three species had not been performed.

The present study compares viability and proliferation of equine, avian, and murine lymphocytes using Lymphoprep™-enriched lymphocyte populations from each species. We also compared the effect of Lymphoprep™ lymphocyte isolation to ACK (ammonium chloride

potassium lysis buffer) lysis buffer on cell recovery, viability, and function of murine splenic lymphocytes. The alamarBlue™ proliferation assays were used to assess the non-specific and mitogen-induced proliferation of the cultured lymphocytes. Trypan-blue exclusion was employed to measure cell viability. Changes in cell viability and death were evaluated with the apoptotic, DNA-binding probe, 7-AAD. Morphological changes were assessed using cytology and a specialized cell counter.

## EXPERIMENTAL

### Mice

Female and male C57Bl/6 mice (6–9 weeks-of-age) were obtained from Charles River Laboratories (Portage, MI) and raised at the Center for Molecular Medicine and Infectious Disease Non Client Animal Facility. All animals were fed a commercial pelleted diet and provided water *ad libitum*. The animals were housed under controlled conditions of temperature (22°C), humidity (40–60%), and lighting (14/10 light/dark cycle). Animal maintenance and care were approved prior to initiation of experiments and, at all times, in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.



### Chickens

Adult white leghorn chickens (14–16 weeks) were obtained from the Virginia Tech poultry farms and maintained in poultry grangers at the Avian Medicine Facility. All birds were fed a commercial pelleted diet (Southern States, Richmond, VA) and provided water *ad libitum*. Animal maintenance and care were approved prior to initiation of experiments and, at all times, in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

### Horses

Adult horses of various ages and genders from the Virginia-Maryland Regional College of Veterinary Medicine's teaching herd were used in the study. All horses were pasture maintained supplemented with a commercial pelleted diet and provided water *ad libitum*. Animal maintenance and care were approved prior to initiation of experiments and, at all times, in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

## Collection and Isolation of Murine Splenic Lymphocytes

Mice were euthanized by cervical dislocation and the spleens were aseptically removed using dissection scissors and curved forceps. Spleens were then placed into prelabeled sterile Petri dishes (Corning, Corning, NY) containing 8 mL of incomplete RPMI-1640 medium (lacking supplements, Mediatech, Herndon, VA). Each spleen was gently dissociated over a stainless steel sieve screen (Sigma, St. Louis, MO) using curved forceps.[1,8,10] Cells were then pipetted through the sieve screen, following dissociation, to remove debris. Cells were washed in incomplete RPMI-1640 for 10 min, 240 × g, and 23°C. The supernatant was then discarded and the cell pellet was resuspended in 8 mL of incomplete RPMI-1640. For the Lymphoprep™ (Oslo, Norway) isolation technique, half of the media cell suspension was carefully added to 4 mL room temperature Lymphoprep™ and centrifuged (30 min, 450 × g, and 23°C). The buffy coats were added to 9 mL room temperature incomplete RPMI-1640 and washed for 7 min, 240 × g, and 7°C. Cell pellets were resuspended in 5 mL incomplete RPMI-1640 and washed a second time and centrifuged (7 min, 240 × g and 7°C). Pellets were resuspended in 5 mL incomplete RPMI-1640 for cellular enumeration. For the RBC lysis technique, the other half of the media cell suspension was centrifuged (7 min, 240 × g and 7°C) and supernatant poured off. The cells were then resuspended in 1 mL incomplete RPMI-1640. To each tube, 3 mL of ACK lysis buffer (pH 7.29) were added, to lyse red blood cells, and tubes were incubated for 5 min at 23°C. After ACK lysis buffer incubation, the cells were resuspended in 5 mL of incomplete RPMI-1640 and washed twice (7 min, 290 × g and 7°C). The splenic leukocyte-rich cells were then resuspended in 5 mL complete RPMI-1640 media containing 10% premium-select pathogen-free low-endotoxin FBS (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/mL penicillin (ICN), and 50 mg/mL of streptomycin (ICN), and maintained between 7–10°C.

## Collection and Isolation of Equine Peripheral Blood Lymphocytes

Approximately 30 mL of peripheral blood were collected from the external jugular vein from each horse into 10 mL heparinized vacutainer tubes. Under aseptic conditions, the blood was diluted 1:2 ratio with incomplete RPMI-1640 at room temperature. A density-gradient, 1:2 dilution of Lymphoprep™ to total volume was made.[7] Briefly, the blood was slowly added, using a pipette, over the density gradient. Once completed, the tubes were centrifuged for 30 min at 450 × g, 23°C. Each buffy coat layer was collected and added to 35 mL room temperature incomplete RPMI-1640 and washed for 7 min, 240 × g, and 7°C. Cell pellets were

then resuspended in 5 mL incomplete RPMI-1640 and washed a second time for 7 min, 240 × g, and 7°C. Cell pellets were resuspended in 5 mL incomplete RPMI-1640 for cellular enumeration.

## Collection and Isolation of Avian Peripheral Blood Lymphocytes

Peripheral blood was collected, with aseptic technique, from the jugular vein with a 23 g needle attached to a 10 mL syringe. The blood was then transferred via an 18 g needle into a sterile heparinized vacutainer tube (Fisher Scientific, Pittsburgh, PA) and mixed by gentle inversion. The tubes, at room temperature, were centrifuged at 50 × g, 10 min, and 23°C. The buffy coat (lymphocyte rich) layer was identified and collected with the use of a gentle "swirl" technique.[9] Briefly, a short 1 mL sterile glass pipette was placed in the plasma above the buffy coat and gently stirred. This created a funnel effect, allowing the lymphocytes to rise off the red blood cell pellet into the plasma. Thus, the plasma and buffy coat suspensions were collected together. The above procedure was repeated at least once. Plasma and buffy coats were diluted 2:1 with room temperature incomplete RPMI-1640. The media cell suspension was carefully added to 4 mL room temperature Lymphoprep™ and centrifuged for 30 min at 450 × g, 23°C. The buffy coats were collected and added to 9 mL room temperature incomplete RPMI-1640 and washed for 7 min, 200 × g, 7°C. Cell pellets were then resuspended in 5 mL incomplete RPMI-1640 and washed a second time for 7 min, 200 × g, and 7°C. Cell pellets were resuspended in 5 mL incomplete RPMI-1640 for cellular enumeration.



## Cell Enumeration

Cells were enumerated and size-analyzed using a Beckman Coulter Multisizer 3® (Fullerton, CA) cell counter according to the manufacturer's protocol. Briefly, a 10 μL aliquot of enriched cell suspension was transferred to a plastic counting-chamber containing 10 mL of PBS (Mediatech). The plastic chamber was capped, mixed by repeated gentle inversion, enumerated and resuspended to $5.0 \times 10^6$ cells/mL.

## Cell Viability Assessment using Trypan Blue Exclusion

A 100 μL aliquot of the cells was diluted 1:5 with trypan blue. Cells were then scored as viable or dead based on stain uptake using a hemocytometer and light microscope (Nikon, Japan), under the 40X objective. Percent viability was assessed for the spleen lymphocytes at the time of isolation, and at 24 and 48 hr.

## Cell Viability and Cell Death Assessment using 7-AAD

Cell suspensions ($5.0 \times 10^6$ cells/mL) at 100 μL per sample were dispensed into individual wells of a 96-well round-bottom tissue culture plate (Corning). 7-AAD fluorescent labels were used according to manufacturer (Pharmingen, CA) recommendation at 0.2 μg/μL. The cells were mixed with PBS (Mediatech) and incubated on ice in the dark for 30 min while mixing on a Gyrotary Shaker Model G2 orbital mixer (New Brunswick Scientific, NJ). Following incubation, the cells were washed at $240 \times g$, 7°C for 7 min and then resuspended in 100 μL of PBS with 7-AAD at 1:10 dilution. Flow cytometry analysis was performed using an Epics XL flow cytometer (Coulter, Miami, FL).[11]

## Cell Surface Marker Expression



Fresh and cultured cells from all time intervals were stained with specific mouse monoclonal antibodies and analyzed by flow cytometry as per our previous studies.[10,12]

Mouse PE-anti-CD4 (Pharmingen, CA), FITC-anti-CD8 (Pharmingen, CA) monoclonal antibodies and Mouse B-cell marker PE-anti-CD45R/B220) (Pharmingen, CA) (0.5 μg/$5 \times 10^5$ cells) were added to splenic cells. The cell antibody suspensions were incubated for 30 min on ice, and then were washed and centrifuged (10 min, $200 \times g$ and 7°C). Cell suspensions were resuspended in 100 μL of PBS, washed, centrifuged, (10 min, $200 \times g$ and 7°C), and evaluated by flow cytometry. Cells were analyzed on an EPIC XL flow cytometer. From each sample, 5000 events were collected and analyzed using the Immuno-4 software program.

## Proliferation Determination using Alamarblue™

One hundred microliters of cells ($5.0 \times 10^6$/mL) were added to triplicate wells of a 96-well round bottom tissue culture plate (Corning, Corning, NY) containing media alone or 10 μg/mL concanavalin A (Con A, Sigma-Aldrich, St Louis, MO) and incubated at 37°C, 5% $C0_2$. At 48 hr of incubation, 20 μL of the alamarBlue™ dye (AbD Serotec, Raleigh, NC) were added to each well and cells were returned to the incubator. At 8 and 24 hr post addition, the degree of absorbance was determined under a dual wavelength (570 and 600 nm) on a Molecular Devices plate reader (Sunnyvale, CA). Mitogen-induced lymphocyte proliferation was determined using the alamarBlue™ dye (AbD Serotec). Duplicate plates were set up for the splenic cell suspension. Following a 48 hr incubation, 20μL of alamarBlue™ dye (AbD Serotec). (10% of

incubation volume) were added to each well of the culture plates. Plates were incubated for an additional 24 hr then measured using a Molecular Devices plate reader.[1,8] In the presence of the alamarBlue™ dye (AbD Serotec), cellular proliferation induces a chemical reduction of the dye, changing the color from blue to red. The intensity of this red color reflects the extent of cellular proliferation. Data were reported as Δ specific absorbance. The value of the Δ specific absorbance was defined as the specific absorbance of the stimulated cells minus the specific absorbance of the cells in media only. Calculations were based on the mean specific absorbance, of the triplicate wells.

## Cytospins

A 100 μL sample of each spleen cell suspension ($5.0 \times 10^6$ cells/mL) was placed into a cytospin chamber containing 100 μL of PBS (Mediatech). Each cytospin chamber consisted of a slide holder, slide, slide filter, and cell cup apparatus. The cytospin chambers were vertically placed into a Cyto-Tek centrifuge (Sakura Finetechnical, Tokyo, Japan) and centrifuged (5 min, $28 \times g$ and 23°C). Cytospin chambers were then removed and the slides were allowed to air-dry overnight. The slides were then stained with a modified Wright Giemsa stain using a Hema-Tek 2000 (Bayer Corporation, Tarrytown, NY) automated stainer as described in previous studies in our laboratory.[12,13]



## Detection of Apoptosis by Light Microscopy of Wright-Giemsa-Stained Cytospin Slides

Standard light microscopy is not a sensitive method to detect apoptosis. However, detecting lymphocyte apoptosis by specific morphologic changes is reliable.[14] The cytologic changes specific to lymphocyte apoptosis include cell shrinkage with condensed nuclei (pyknosis), and nuclear fragmentation (karyorrhexis). Employing light microscopy, apoptotic cells were identified by an investigator who was blinded to sample identity, by assessing pyknosis and karyorrhexis with oil immersion objective (×1000 magnification) in each cytospin slides stained with Wright Giemsa stain.

## Statistical Analysis

Data are presented as arithmetic mean ± standard error of the mean (SEM). The group size was 4 animals/species for each experiment. The

species comparison study (horse, chicken. mouse) consisted of three replicate experiments. The cell lysis buffer vs. Lymphoprep™ studies consisted of 4 mice per experiment and was replicated with two experiments. A Student's *t*-test was used to test for significance among groups for the cell lysis buffer vs. Lymphoprep™ study. Results are described as significantly different at $p < 0.05$.

## RESULTS AND DISCUSSION

### Coulter Cell Enumeration

#### Murine Lymphocyte Isolation Study

*ACK Lysis Technique Versus Lymphoprep™.* The average total cellular recovery from each mouse spleen was $82.4 \pm 6.0 \times 10^6$. The cell suspensions from these spleens were split in half for each enrichment technique. These average initial concentrations were determined to be $41.2 \pm 3.1 \times 10^6$ cells. The average yield of mouse splenic lymphocytes enriched by the density-gradient technique was $11.2 \pm 1.7 \times 10^6$ cells (>92% purity) that equated to a 27.1% recovery of the loading concentration. The average yield of splenic lymphocytes by RBC lysis technique was approximately $29.0 \pm 5.5 \times 10^6$ cells (>86% purity) or a 70.4.0% recovery of average initial concentrations.

#### Avian, Equine, Mouse Lymphoprep™ Study

The average yield of equine peripheral blood lymphocytes from ~30 mL of heparinized blood was $30.2 \pm 3.6 \times 10^6$ cells with greater than 90% purity. The average yield of mouse splenic (~70 mg spleen) lymphocytes enriched by density-gradient technique was $26.5 \pm 4.8 \times 10^6$ cells with greater than 90% purity. The average yield of avian peripheral blood lymphocytes from ~8–10 mL of heparinized blood was $80.9 \pm 15.4 \times 10^6$ cells with greater than 94% purity.

### Viability with Trypan Blue Exclusion

#### Lymphocyte Isolation Study

*Lysis technique versus Lymphoprep™.* Cell viability and cell death at 0, 24 and 48 hr were measured by trypan blue exclusion in murine lymphocytes isolated by ACK lysis technique and density-gradient technique. The average murine density-gradient enriched lymphocyte viability at 0 hr

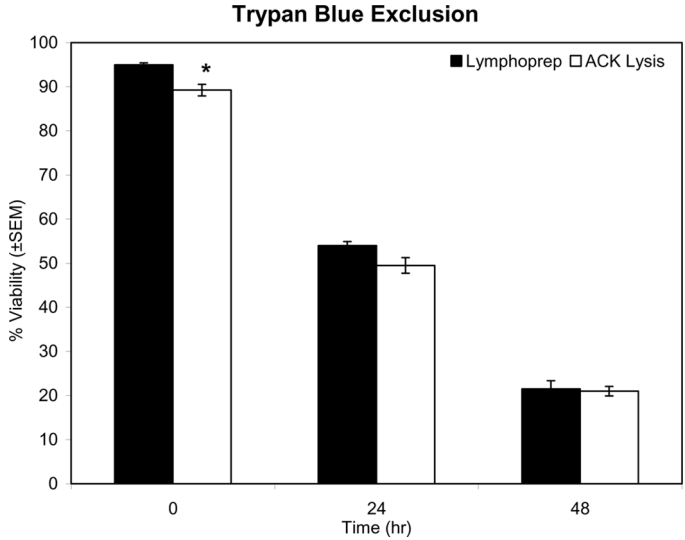


**Figure 1.** Trypan Blue Exclusion: Mouse spleen cell suspensions were evaluated at collection 0, 24, and 48 hr in culture for murine spleen only. Cells were diluted 1:10 and enumerated on a hemacytometer. Data are reported as viable cells ± SEM. ($^{*} = p \leq 0.05$, N = 4 mice/ experiment × 2 experiments).



of culture was approximately 93% compared to 88% for lysis technique enriched lymphocytes. Murine lymphocyte viability declined to about 55% at 24 hr and 20% at 48 hr regardless of the technique used (Figure 1)

Avian, Equine, Mouse Lymphoprep™ Study

Cell viability and cell death at 0, 24 and 48 hr were measured by trypan blue exclusion in equine, murine and avian lymphocytes. At 0 hr of culture, equine and avian lymphocytes had average viabilities that were greater than 93%. The average murine density-gradient enriched lymphocyte viability at 0 hr of culture was approximately 92%. Equine viability was approximately 83% at both 24 and 48 hr of culture. Avian lymphocyte viability was approximately 83% at 24 hr and 70% at 48 hr. Murine lymphocyte viability declined to about 55% at 24 hr and 20% at 48 hr (Figure 2).

## Viability, Apoptosis and Late Apoptosis/Necrosis with 7-AAD

*Murine Lymphocyte Isolation Study: RBC Lysis Technique versus Lymphoprep™.* Cell viability, apoptosis and late apoptosis/necrosis

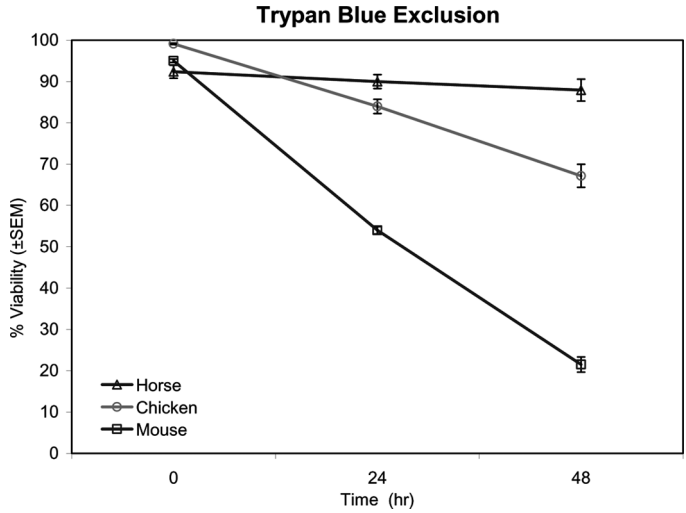


**Figure 2.** Trypan Blue Exclusion: Enriched lymphocytes from the horse, mouse and chicken were assessed for viability at 0, 24 and 48 hr of culture. Cell suspensions stained with trypan blue were enumerated by light microscopy using a hemocytometer. Cell viability was determined by dividing the number of viable cells by the total number of viable cells and dead cells (% $\pm$ SEM) (N = 4 animals/species/experiment $\times$ 3 experiments).



were measured at 0, 24 and 48 hr by 7-AAD in murine lymphocytes enriched by ACK lysis technique ordensity-gradient technique. At 0 hr of culture, the average murine lymphocyte viability was approximately 81% for density-gradient technique and 71% for lysis buffer technique. However, murine lymphocyte viability declined to about 55% at 24 hr and 21% at 48 hr regardless of the technique used (Figure 3).

## Avian, Equine, Mouse Lymphoprep$^{TM}$ Study

Lymphocyte cell viability, apoptosis and late apoptosis/necrosis at 0, 24 and 48 hr were measured with the 7-AAD dye. At 0 hr of culture, equine and avian lymphocytes had average viabilities that were greater than 95%. The average murine lymphocyte viability at 0 hr of culture was approximately 81%. Equine lymphocyte viability was approximately 85% at both 24 and 48 hr of culture. Avian lymphocyte viability was approximately 83% at 24 hr and greater than 73% at 48 hr. Murine lymphocyte viability declined to about 55% at 24 hr and 21% at 48 hr (Figure 4).

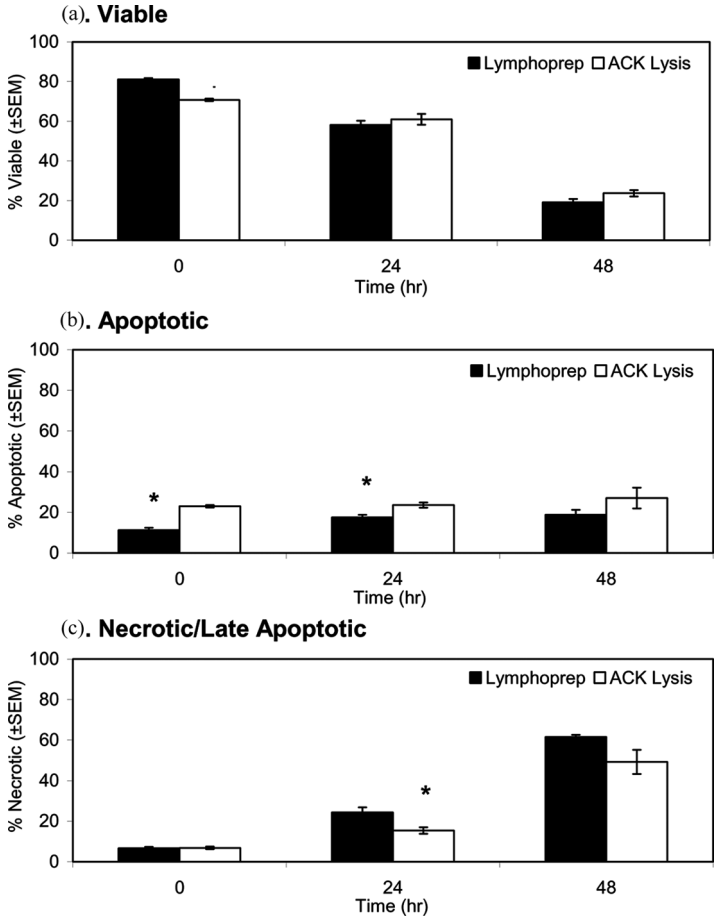


**Figure 3.** Viability determination of splenic lymphocytes cultured for 0 hr, 24 hr, and 48 hr. Cultures were analyzed by flow cytometry analysis with 7-AAD. Analysis detected normal live, apoptotic, and necrotic cells at 24 hr and 48 hr culture. Viability (**a**), apoptosis (**b**) and late apoptosis/necrosis (**c**) were assessed based on size, granularity and uptake of 7-AAD with a EPICS XL flow cytometer (5000 events/sample) ($^* = p \leq 0.05$, N = 4 mice/ experiment × 2 experiments).

## Cell Surface Marker Expression

### Murine Lymphocyte Isolation Study

*ACK Lysis Technique versus Lymphoprep*$^{TM}$. The ACK lysis technique and density-gradient technique showed significant difference in the percentage of lymphocyte subpopulations. The percentage of B-cells

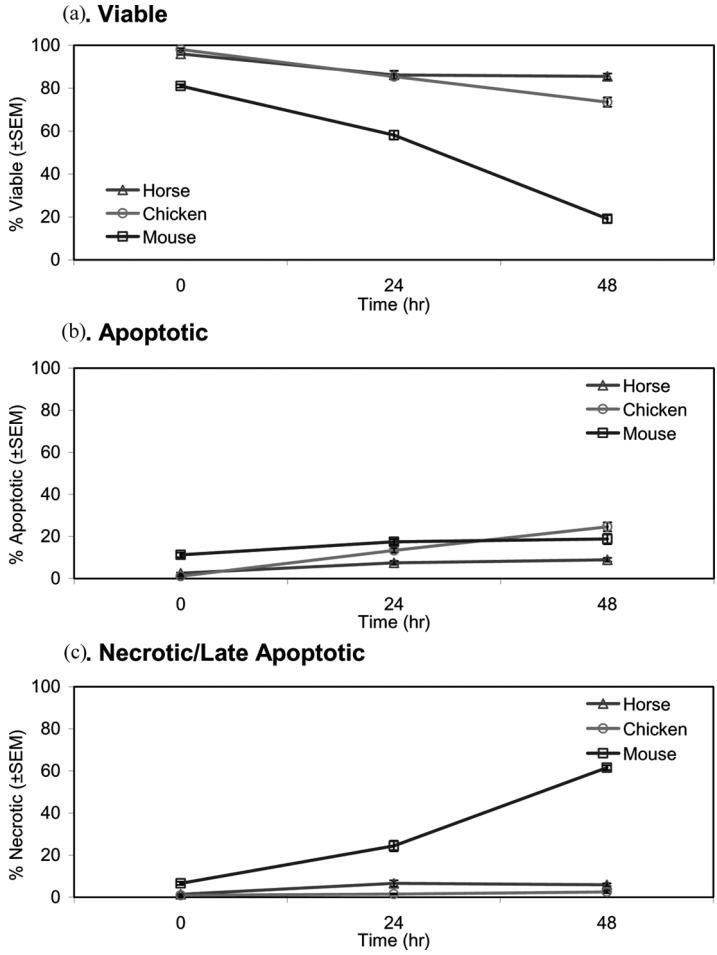


**Figure 4.** Cellular viability, apoptosis and necrosis, 7-AAD. Enriched lymphocytes from the horse, mouse and chicken were assessed for viability at 0, 24 and 48 hr of culture. Cell suspensions (fresh and cultured) were washed with incomplete media, and stained with 7-AAD for 30 min at 7°C, in the dark. Viability (**a**), apoptosis (**b**) and late apoptosis/necrosis (**c**) were assessed based on size, granularity and uptake of 7-AAD with an EPICS XL flow cytometer (5000 events/sample) (N = 4 animals/species/experiment × 3 experiments).

was significantly higher in lymphocytes enriched by density-gradient technique compared to lymphocytes enriched by ACK lysis technique. However, percentage of T-cells ($CD8^+$, $CD4^+$) was significantly lower in density-gradient isolated lymphocytes compared to RBC lysis isolated lymphocytes. Density-gradient isolated splenic lymphocyte surface antigen expression for $CD8^+$ or $CD4^+$ T cells, and B cells

(CD45R/B220$^{+}$) were approximately 6%, 12%, and 75%, respectively Whereas, RBC lysis isolated splenic lymphocyte surface antigens were approximately 14%, 20% and 55%, respectively. The percentages of lymphocyte surface antigen expression were consistent in both techniques and at all time intervals (Figure 5).

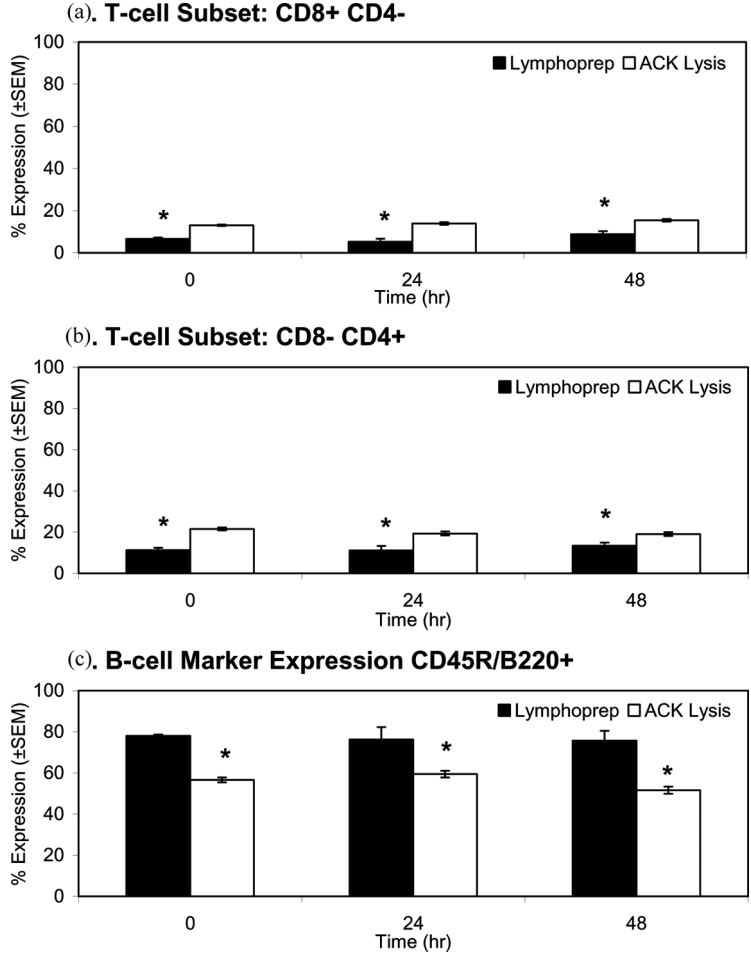


**Figure 5.** Enriched murine splenic lymphocytes from RBC lysis technique and density-gradient technique were analyzed for lymphocyte surface antigen expression at 0, 24 and 48 hr of culture. CD4 (**a**), CD8 (**b**) and CD45R/B220 (**c**) surface antigen expression was assessed with an EPICS XL flow cytometer (5000 events/sample). Data are reported as lymphocyte expression $\pm$ SEM. ($^{*} = p \leq 0.05$, N = 4 mice//experiment $\times$ 2 experiments).

## Spontaneous Proliferation and Con A-Induced Proliferation

The results from the proliferation experiments showed that equine peripheral blood lymphocytes consistently had the highest rate of spontaneous proliferation. The avian lymphocytes were considerably lower. Surprisingly, murine lymphocytes had the lowest spontaneous proliferation regardless of the technique used (Figure 6). However, Con A-induced proliferation varied according to lymphocyte isolation technique used. Murine lysis-treated splenic cell suspensions cultured for 72 hr in 10 μg/mL Con A had greater Δ absorbance than density-gradient enriched lymphocytes, $0.73 \pm 0.097$ and $0.62 \pm 0.099$, respectively.

## Cytologic Analysis

Cytologic specimens revealed large numbers of apoptotic lymphocytes with compacted or fragmented nuclei. The apoptotic figure was usually represented as a cluster of chromatin-condensed nuclei within a



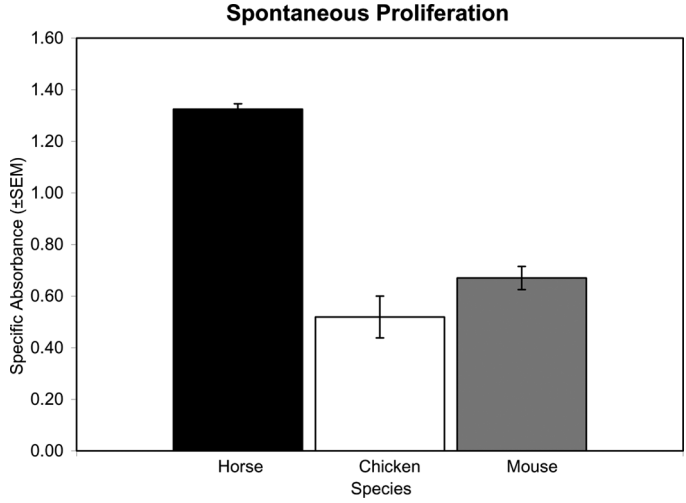


**Figure 6.** Spontaneous proliferation. Enriched lymphocytes from the horse, mouse and chicken were cultured in complete RPMI-1640 media for 72 hr. At 48 hr of culture, 20 μL of alamarBlue™ were added to each well containing 200μl cells and media of a 96-well tissue culture plate. At 72 hr of culture, the plates were removed and evaluated for degree of color change that correlates with proliferation level. Values are reported as mean specific absorbance ±SEM, (N = 4 animals/species/experiment × 3 experiments).

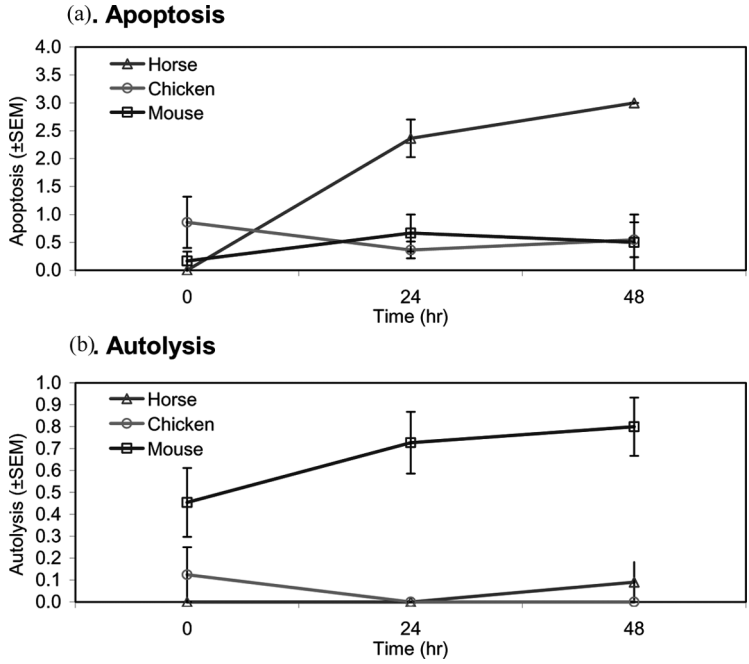


**Figure 7.** Cytologic analysis of apoptosis and autolysis. Cytospin slides of enriched lymphocytes from fresh and cultured equine, mouse and chicken leukocytes (0, 24, & 48 hr) were prepared and analyzed by a licensed pathologist. Cells were scored (0–4) based on incidence of apoptosis (**a**) and (0–1) based on severity of autolysis (**b**). The values are reported as means ±SEM, (N = 4 animals/species/experiment × 3 experiments).



condensed cell cytoplasm. Sometimes, the apoptotic figure was simply a cluster of cell rounded fragments of various sizes, each containing a chromatin-condensed nuclear fragment. Semi-quantitative scoring of the samples showed increased apoptosis over time in culture (Figure 7a). The horse cells had the lowest degree of apoptosis at time 0. Marked autolysis (dead cells) was noted in the murine cells from time 0 to 48 hr (Figure 7b).

## CONCLUSION

It is not uncommon for multiple laboratories conducting research in the same area to publish contradictory results. This discordance in data may in some cases center around differences in the methodologies performed by the researchers. For instance, we recently found that spleen

dissociation technique significantly affects mitogen-induced T-cell proliferation in culture.[1] The only functional endpoint evaluated in this study was lymphocyte proliferation. It remains unknown if additional endpoints, such as cytokine production, may underlie the observed functional difference. The current study had two goals. The first was to determine if identical lymphocyte enrichment techniques and culture conditions resulted in equivalent viability and function across three research species routinely used in our laboratory. The second goal was to compare the effects of the cell lysis buffer enrichment technique and the standard density-gradient technique on murine splenic lymphocyte phenotype, function and viability.

Methods of lymphocyte enrichment tend to vary across species primarily due to optimization requirements. Typically, the cell lysis separation method is employed to enrich murine splenocytes due to its simplicity, good viability and high yield.[1] Slow spin separation of heparinized avian peripheral blood yields high recovery and viability for chicken lymphocytes.[9] Density-gradient collection techniques are widely employed for collection of lymphocytes from peripheral blood of many mammalian species (i.e. equine). Particularly, Ficoll-sodium diatrizoate and Percoll are the most commonly used gradients to separate equine blood lymphocytes and granulocytes, respectively.[15,16] However, density-gradient purification is tedious and requires technical skills to place diluted blood over the gradient as well as to collect the separated fractions. In addition, several factors (cell content, volume and dilution of the sample; pH, density and osmolarity of the gradient; time, gravity and temperature of centrifugation) are known to influence the results of a density-gradient separation and this limits the reproducibility of the method.[15] For example, we previously observed that changing the centrifugation speed and time alters the total recovered lymphocytes (unpublished data). As shown in this study, density gradient separation methods are cell wasting. For example, depletions in present mouse splenic lymphocyte recovery of up to 50% occurred compared to the lysis buffer enrichment technique.

Trypan blue exclusion is a relatively simple and common technique to enumerate live from dead cells using light microscopy. It is limited in that it cannot stage nor distinguish apoptotic cells from necrotic cells and there is some degree of subjectivity in the enumeration process. Still, it is a rapid, inexpensive technique used by many laboratories for measuring cell viability. It is less sensitive than analysis with cell death dyes via flow cytometry.[17] In the present study, we used the trypan blue exclusion assay to determine the viability of the cell cultures at 0 hr, 24 hr, and 48 hr. Differences in viability at the different time intervals were readily noted among the lymphocytes of the three species. At 0 hr of culture, both equine and avian lymphocytes were greater than 93% which is what

we have observed in previous studies.[7,9] The 0 hr viabilities of the lysis-enriched cells and density-gradient enriched mouse lymphocytes were approximately 88% and 93%, respectively. In our previous murine studies using lysis buffer enrichment, viabilities as measured by trypan blue exclusion were commonly in this range.[8,10] At 24 hr of culture, the equine lymphocytes maintained the highest viability followed by chicken lymphocytes then the murine lymphocytes. By 48 hr, equine lymphocyte viability remained high at about avian lymphocyte viability declined by 30% from 0 hr viability and murine lymphocyte viability declined by 70% from the 0 hr viability. When the murine study comparing the Lymphoprep™ to lysis buffer was performed, the same decreasing trend in viability was observed at all three time points. This is an observation we have seen consistently with murine mortal cell cultures.

The DNA-binding probe, 7-AAD, allows for assessment of the relative percentages of cells that are viable, early apoptotic, or late apoptotic/necrotic via flow cytometry.[11,14,18] In this study, viability characterization by 7-AAD showed the same profile of results as the trypan blue stain. At all three times of evaluation, the murine lymphocytes had lowest viabilities and higher percentages of early apoptotic cells and late apoptotic/necrotic cells than either the equine or avian samples. As previously reported, the percentage of viable cells across all groups was lower by 7-AAD compared to trypan blue, suggesting that the trypan blue exclusion method cannot distinguish live cells from cells in early stages of apoptosis.[17,19]

The significant species differences in lymphocyte viability under identical culture conditions were reflected in the rate of spontaneous proliferation as measured by alamarBlue™. Equine lymphocyte proliferation was on average three times higher than the avian lymphocyte spontaneous proliferation. Murine lymphocytes had the lowest spontaneous proliferation. This differential proliferation outcome was interesting since the enrichment and culture conditions used to procure lymphocytes of these three animal species were identical. This would suggest that reactivity of T lymphocytes to Con-A is different across these three species.

The murine lymphocytes again showed evidence of enhanced cell death in cytologic preparations, as indicated by "ghosting" or autolysis on the slides. In general, the avian and equine cytospin slides showed higher numbers of discernable lymphocytes *in vitro* than the murine model throughout the culture duration.

Lymphoprep™ appeared to cause some toxicity to murine splenic lymphocytes, in the form of reduced viability and Con-A induced proliferation. We therefore compared the influence of density gradient (Lymphoprep™) enrichment to the cell lysis buffer technique on the

cellular recovery, lymphocyte cell subset marker expression and non-specific proliferation. The two techniques differentially influenced splenic lymphocyte recovery, cell subset phenotypes, and lymphocyte function. Lymphocytes enriched with the cell lysis buffer technique were on average 24% higher than the density gradient technique. The density gradient yielded higher viabilities at 0 hr, an observation that would be expected since density gradient enrichment is designed to remove dead cells from a cell suspension. By 24 and 48 hr, cell viability and death were comparable across both enrichment techniques.

As suggested above, lymphocyte surface antigen expression was different by isolation technique. The percentage of B-cells (CD45R/B220) was higher while both mature T-cell subpopulations ($CD4^+$ or $CD8^+$) were lower at 0 hr in cells enriched by the density gradient technique. The percentage of expression of the T cell subsets and B cells in lymphocytes enriched by the cell lysis buffer were consistent with previously published studies.[8,10] Technique-related differences in percentages of T cell and B cell lymphocyte surface antigen expression were also observed at 24 and 48 hr. These data suggest that the enrichment of splenic lymphocytes using Lymphoprep$^{TM}$ disproportionately depletes T cells and enriches B cells.

Average Δ absorbance in Con A-induced lymphocytes showed proliferation for the cell lysis enriched cells to be higher than the density gradient enriched lymphocytes. A likely explanation for this observation is that the density gradient yielded lymphocytes that were significantly depleted of T cells, which predictably would result in a weaker Con A proliferative response. This depletion of T cells via the Lymphoprep$^{TM}$ could also be due to differences in cell density between the subsets and needs to be further explored. Alternately, it is possible that each isolation technique may differentially affect cytokine production or secretion, which in turn alters in vitro proliferation. Whether cytokine synthesis, response, or expression is sensitive to the method of lymphocyte isolation warrants further investigation.

In summary, results from this study show that under similar enrichment procedures and culture conditions, lymphocyte viability and function vary significantly among species. Also, for murine lymphocytes, the choice of lymphocyte enrichment technique affects numerous immunologic endpoints, and as such could affect interpretation of data.



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