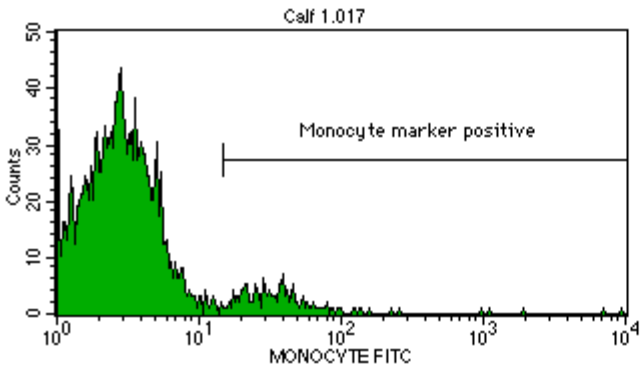
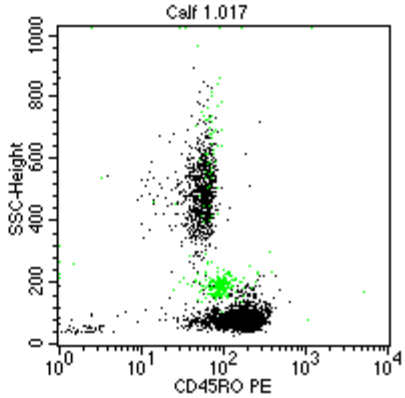
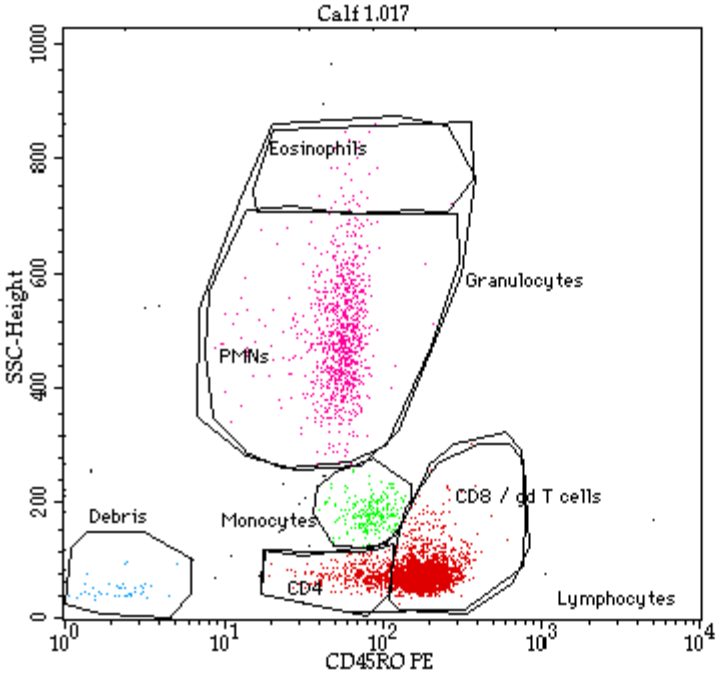
Two color plots were originally set up to check relative location of leukocyte subsets on the CD45 / SSC plot. Gating on the positive cells on the histogram shows the location of those cells in green on the 2 dimensional plot.





The CD45 / Side Scatter plot can then be used to generate statistics on the differential. The plot shows a breakdown of the lymphocytes in approximate CD4 and CD8 /λδ T cell populations, as well as eosinophils separate from PMNs. The lymphocyte subset percentages are approximate due to overlap of the populations.

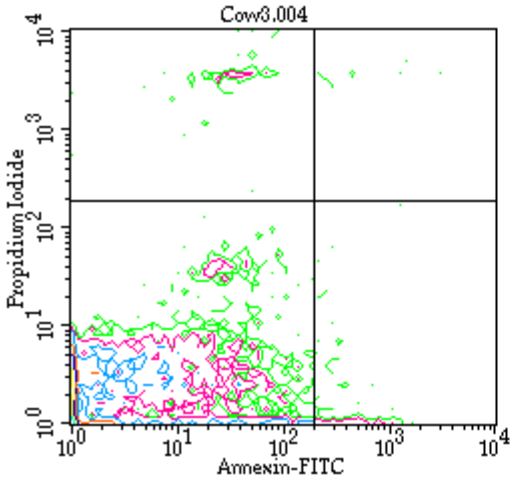


Total Events: 5000
X Parameter: FL2-H CD45RO PE (Log)
Y Parameter: SSC-H SSC-Height (Linear)

Region	Events	% Total
Lymphocytes	3723	74.46
CD4	406	8.12
CD8 / gd T cells	3335	66.70
Monocytes	230	4.60
Granulocytes	971	19.42
PMNs	938	18.76
Eosinophils	30	0.60
Debris	40	0.80

Results:

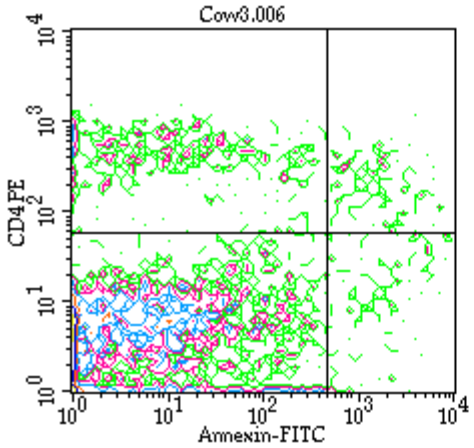
A plot showing propidium iodide and Annexin is used to determine apoptotic (Annexin only +) from necrotic (PI+ and Annexin +). This group should be a low percentage. Surface markers can then be used to select sub populations for Annexin positive results, indicating apoptosis.



Gate: No Gate
Gated Events: 5000
Total Events: 5000
X Parameter: FL1-H Annexin-FITC (Log)
Y Parameter: FL2-H Propidium Iodide (Log)

Quad	Events	% Gated	% Total
UL	242	4.84	4.84
UR	94	1.88	1.88
LL	4413	88.26	88.26
LR	251	5.02	5.02

Annexin / propidium iodide contour plot shows: ~2% of population dead (PI+ and Annexin+), ~5% Annexin + (apoptotic). The ~5% PI positive are assumed to have died in the final steps of the reaction, after the Annexin has been diluted.

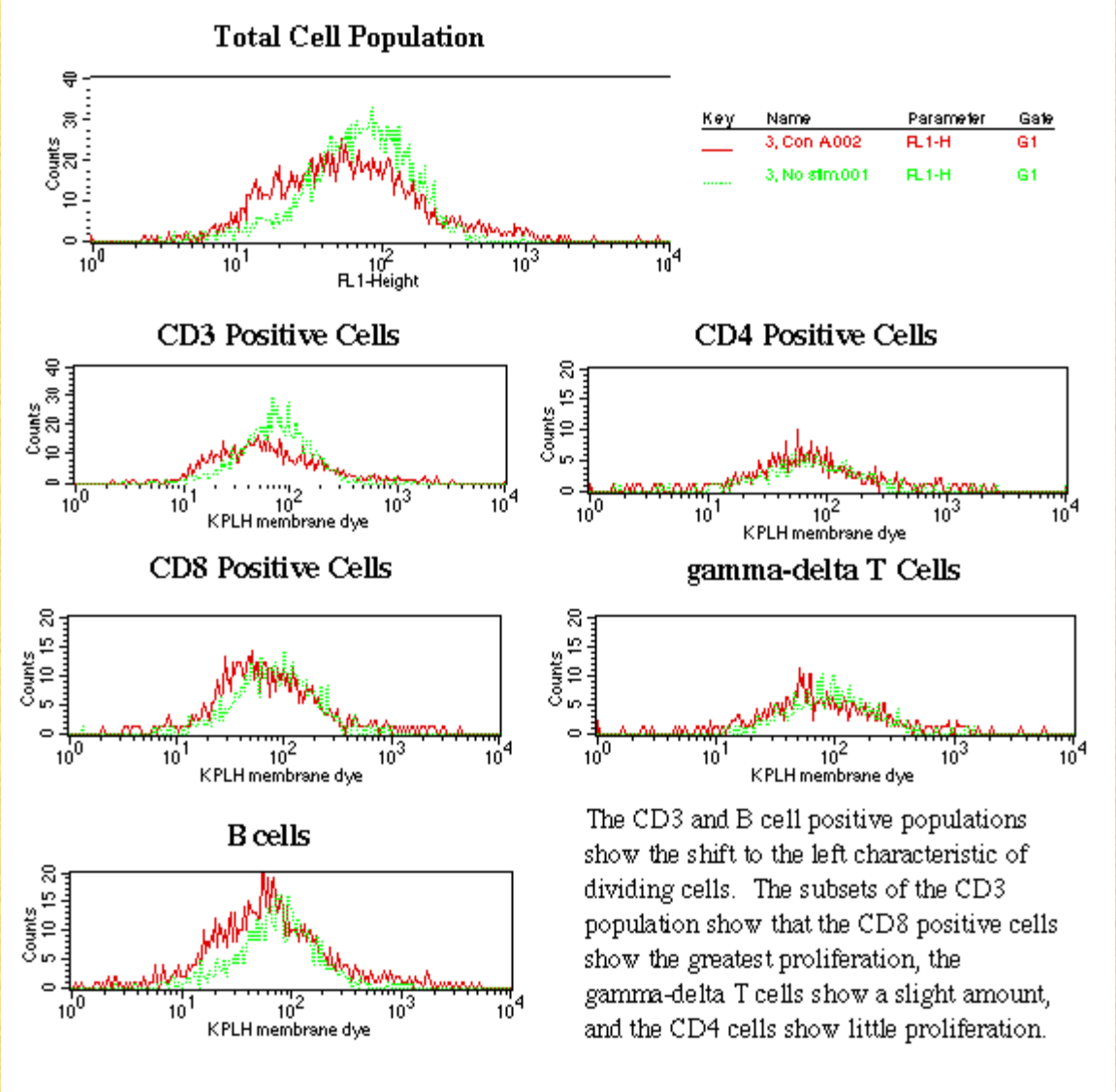


Gate: G1
Gated Events: 3207
Total Events: 5000
X Parameter: FL1-H Annexin-FITC (Log)
Y Parameter: FL2-H CD4 PE (Log)

Quad	Events	% Gated	% Total
UL	425	13.25	8.50
UR	121	3.77	2.42
LL	2541	79.23	50.82
LR	120	3.74	2.40

CD4 and Annexin: 3.77 percent of the total population are double positive, or ~20% of the total CD4+ population are showing signs of apoptosis.

Results:
An overlaid histogram shows the shift to the left, of the Concanavalin A stimulated population (in red) after 96 hours in culture. This indicates a lower amount of dye in the membrane as it is diluted by divisions of the cell. Subsets can also be examined individually for detection of proliferation if surface labeled prior to analysis.



G00081

New flow cytometric techniques for the analysis of blood from large animals

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Abstract:
A variety of newly developed flow cytometric techniques adapted for the bovine are described. Among these are: CD45 labeled / side scatter comparison for doing differentials in whole blood; apoptosis (programmed cell death) as determined using Annexin V-FITC; PKH2 (a green lipophilic membrane dye) determination of cell proliferation; and 3-color assays using PerCP and PE in conjunction with the Annexin and PKH2 techniques.

Introduction:
Introduction:

The flow cytometer is a relatively new instrument in the veterinary research field. It combines high throughput with sensitive light detection instrumentation to record laser light scatter and fluorescent properties on a per cell basis. This enables the researcher to examine relative size, complexity and up to three or more fluorescent properties on individual cells from a single cell suspension.

Common uses for the instrument have been to label the surface of the cell with fluorochrome labeled antibodies and then examine the cells for fluorescence or to stain the DNA and do cell cycle analysis. New techniques are being developed constantly. The capabilities are there to do internal cell pH, ion flux, internal cellular cytokine production, and a myriad of other new techniques.

Three new techniques from other research arenas, that we have adapted to the bovine, are shown here. These include: a CD45 (leukocyte common antigen) based differential count on whole blood; a three color apoptosis (programmed cell death) detection assay using Annexin V; and a cell subset proliferation assay using a lipophilic green membrane dye, PKH2.

Materials and Methods:
CD45 Whole Blood Differentials:

Slide based differentials in the bovine are tedious and inconclusive due to the difficulty in differentiating monocytes from lymphocytes without an esterase stain. Combining CD45, the leukocyte common antigen, binding properties with the 90o light scatter properties of a cell enables one to accurately determine the percentages of leukocyte types in a whole blood sample.

Materials and Methods:
96 well, round bottom plates (Costar, Cambridge, MA) are preloaded with: 10ul of murine anti-bovine CD45 (VMRD, Pullman, WA) clone CACTB51A at 25mg/ml and 10ul of goat anti-mouse IgG2a-PE (Southern Biotechnology Associates, Birmingham, AL). An isotype or negative control should be done for each sample. These plates can be prepared ahead of time and stored sealed, at 4 degrees C for up to 2 weeks.

50ul of whole blood, collected in ACD as an anticoagulant, is added per well. Plates are incubated for 10 minutes in the dark at room temperature or for 5 minutes at 39 degrees C. Following incubation, red blood cells are lysed with 200ul of cold, distilled water for 10 seconds, followed by 20ul of 10 X NaCl to restore isotonicity. Plates are then centrifuged for 2 minutes at 400 x G. Supernatant is dumped and cells are resuspended in 200ul of FacsLyse (Becton Dickinson, San Jose, CA). Incubation is for 10 minutes in the dark. Samples are then read on the flow cytometer.

Annexin V Determination of Apoptosis:

Apoptosis is also known as programmed cell death. It is the process by which cells remove themselves from the system, either by stimulation from an outside source, or through natural processes. Annexin V binds to phosphotidyl serine. Phosphotidyl serine is normally expressed only on the cytoplasmic side of the cell membrane. In apoptotic cells, the membrane starts to invert, and the phosophotidyl serine can be detected on the external side of the membrane. The Annexin is conjugated to FITC and thus the percentage of apoptotic cells can be determined within a subset with simultaneous surface marker labeling.

Materials and Methods:
96 well round bottom plates (Costar, Cambridge, MA) are preloaded with 75ul each of the primary antibodies of interest. These are at concentrations from 3.5-14 ug/ml. 100ul of whole blood in ACD anticoagulant is added to each well and incubated at room temperature for 15 minutes. Plates are centrifuged at 400 x G for 2 minutes and plasma decanted. Red blood cells are lysed with 200 ul of cold distilled water for 10 seconds followed by restoration of isotonicity with 20ul of 10 X NaCl. Plate is centrifuged as before. This step is repeated. 75ul of appropriate secondary antibodies are added and incubated as before. After centrifugation, cells are treated as per the instruction, (Annexin kit, R & D Systems, Minneapolis, MN) except 10ul of Annexin at half of the concentration of the R&D protocol is added to each well. Plates are incubated for 10 minutes and transfered to 12x75mm tubes (Falcon, Becton Dickinson, Lincoln Park, NJ) during this time. Tubes are then diluted with 400ul of binding buffer and read on the flow cytometer.

Cell Proliferation Studies using PKH2

Various techniques have been used to study proliferation of cultured cells. This method offers the researcher the ability to directly determine which subsets are dividing in culture. Cells are labeled, preculture, with PKH2, a non-toxic lipophilic dye, which fluoresces green with argon laser stimulation. The dye quantity is diluted as subsequent generations share the dyed membrane fractions. While, ideally, one should be able to track subsequent generations as each should have half of the amount of dye as the preceding generation, in the mononuclear blood cell preparation that we use, this has not been possible.

Materials and Methods:
Mononuclear cells are isolated from blood, either by buffy coat harvest or density gradient centrifugation. Cells are adjusted to 2 x 10e9/ml in kit supplied diluent (Sigma Kit PKH2-GL, Sigma, St. Louis, MO). An equal volume of PKH2 dye is added at a 4 x 10e-6M concentration. Mix gently and incubate at room temperature for 5 minutes. Add an equal volume of 100% serum or other protein solution to stop the reaction. Incubate for 1 minute. Dilute with another equal volume of complete medium of choice. Centrifuge and wash three times in complete medium. 5 x 10e5 cells are added to a total volume of 200ul of RPMI 1640 (Gibco, Grand Island, NY) with 5% fetal bovine serum and 1% antibiotic-antimycotic, (Sigma, St. Louis, MO) in 96 well, flat bottom plates (Falcon, BD Labware, Lincoln Park, NJ). Populations are cultured for 96 hours in 5% CO2 at 39 degrees C with and without 4ug/ml of Concanavalin A (Sigma). Like wells are pooled and surface labeled and examined on the flow cytometer.

Results:
CD45 Differential Results: (see figure 1)
A two dimensional plot , representing CD45 uptake on the X-axis and 90 degree scattered light (an indication of the cell¹s internal complexity) on the Y-axis is shown. Each cell population, including monocytes, can be found in a

discrete region, which makes this technique superior to a manual differential. The statistical significance is also enhanced due to the fact that this technique examines 5000 cells, rather than the usual 100-200 cells examined on the microscope.

Annexin Results: (see figure 2)
A plot showing propidium iodide and Annexin is used to determine apoptotic (Annexin only positive) from necrotic (PI and Annexin double positive). This should be a low percentage. Surface markers can be used to select populations to examine for Annexin positive populations.

PKH2 Results: (see figure 3)

Discussion and Conclusions:
Conclusions:

The CD45 based differential assay has become a routine tool for human diagnosticians in leukemia research, and has now been adapted to serve the large animal research community. It provides fast preparation and analysis times with the ability to examine large numbers of cells. It eliminates the laborious task of reading large numbers of slides and provides a reliable means of easily defining the monocyte population.

Understanding apoptosis and what triggers it, is the subject of study in many research labs. The ability to make the determination of apoptotic cells with Annexin, without the traditional gel preparation steps, along with the ability to simultaneously look at individual subsets of a population is a critical step in this research area.

Various assays have been forwarded in an attempt to replace tritiated thymidine incorporation assays and the associated radioactivity. The PKH2 assay is another step in that direction. In our hands, this method shows that proliferation occurs and more importantly, which subsets are responding. With further work, it may be possible to quantify the responding subsets as to the numbers of generations and generation times. Propriatary software is available from Sigma to do this.

References:

Comments:

Address questions and comments about this abstract to Bruce Pesch (bpesch@nadc.ars.usda.gov).

Previously presented at Association of Laboratory Technicians "Sixth Annual Symposium" on January 28, 1997.

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Questions and Answers

Q: The CD45 differential is really intersting. In the figure legend I can't see the b-lymphocytes. Where would they localise on the plot?

A:The B cells were not pictured in the CD45 figures. They tend to localize in the same areas as the lymphocytes, but are scattered throughout the area. They do not clump in a single spot. In our experience they are a low enough percentage in the normal animal that the B cells will not change the T cell subset profile substantially, and conversely, it is difficult to directly quantitate the B cells on the CD45 - 90 degree light scatter plot.

Q We've been using flow cytometry for doing leukocyte differentials on cattle and sheep for about a year now. My methods are not quite as sophisticated as the one from your group - I use a double stain CD45 and CD14 and identify classes of leukocytes by CD14 versus SSC plot for a 4 part diff. Results correlate well with manual diffs except that there are more monocytes (CD14+ cells) by flow cytometry than by microscope.

A: We have made a similiar conclusion with our data. We developed this technique of performing differential cell counts because we were not satisfied with the accuracy of differential cell counts performed by light microscopy. We in fact, cite the work by Hammer RF & Weber AF (1974. Ultrastructure of agranular leukocytes in peripheral blood of normal cows. Am J Vet Res 35:527-536), whenever we report differential data, as our reason for pooling monocytes and lymphocytes together as mononuclear cells. In Hammer's paper, they found that differential counts of agranulocytes in bovine blood on the basis of ultrastructural differed widely from conventional hematology results. They too found a greater % of monocytes when differential's were performed on the basis of ultrastructural characteristics (22.3% monocytes vs 6.5% by light microscopy). Obviously, transmission EM is not practical as a routine method to perform a differential cell count on bovine blood, yet it is undoubtedly far more accurate in the identification of leukocyte subsets. Hammer concluded that this discrepancy is likely due to the wide range of sizes of bovine lymphocytes and that the majority of bovine monocytes may actually be mis-classified as large lymphocytes when identified by light microscopy. More than 10 years ago, we attempted to develop esterase staining techniques for the purpose of accurately quantitating circulating monocytes, but found a wide range of esterase staining intensity and felt that the esterase stains were subject to wide technical variation. We suspect that your observation of increased monocytes in your differentials are consistent with the findings of Hammer and ourselves, in that routine hematological techniques, woefully underestimate the number of monocytes in bovine blood. We cannot speak for your findings in sheep, but it would not be a great leap of logic to assume a similar situation exists in other ruminants. Your technique in combining staining for CD45 with CD14 is something we considered, but decided that we got equal information with CD45 vs SSC alone, and it was cheaper to do CD45 alone. There may be situations that you have found where CD14 adds to the analysis. We'd be interested to know if you've found any.

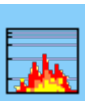
Figure 1

Figure 2

Figure 3