



Evaluation of Drug-Induced Alterations in Lymphoid Tissues by Object Oriented Image Analysis using Virtual Imaging

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ABSTRACT

This work compares the quantitation of changes in B- and T-cell populations of rat lymphoid tissue obtained using Definiens eCognition software with Nanozoomer virtual images to results obtained using laser-scanning cytometry (LSC). Male Sprague-Dawley rats received intraperitoneal injections of sterile saline (vehicle) or cyclophosphamide (6 mg/kg) for 14 days to induce immunosuppression. On day 10, rats received keyhole limpet hemocyanin (KLH) (100 µg/animal) intravenously to induce a T-cell dependent antibody response. Animals were sacrificed on day 15, and spleen and thymus organ weights were recorded. Anti-KLH IgM endpoint titers were measured using an enzyme-linked immunosorbent assay (ELISA). Formalin-fixed, paraffin-embedded samples of spleen, thymus and mesenteric lymph node were routinely processed and stained with hematoxylin and eosin for histologic evaluation by a pathologist and fluorescent and chromogenic staining for T-cell (CD3) and B-cell (CD79ac) populations for assessment with LSC and a custom Definiens ruleset respectively. Cyclophosphamide treated rats had a mean 98% decrease in anti-KLH IgM response, decreased splenic and thymus weights (30% and 25%, respectively), and histopathological evidence of lymphoid depletion in the spleen. Neither Definiens nor LSC analysis of T-cell populations revealed significant differences in the spleen or mesenteric lymph nodes in vehicle vs. treated animals. Examination of B-cell populations revealed significant decreases in the spleen (29.5 ± 3.1 vs. 10.3 ± 1.4 by LSC and 37.9 ± 3.5 vs. 16.0 ± 1.1 by Definiens) and mesenteric lymph nodes (12.9 ± 2.7 vs. 3.8 ± 0.9 by LSC and 19.8 ± 4.1 vs. 8.6 ± 1.8 by Definiens) of treated animals. In summary, image analysis of virtual images using Definiens eCognition software can be used to quantitate T- and B-cell populations in rat lymphoid tissues and these data correlate with LSC data and serologic evidence of decreased IgM.

INTRODUCTION

Immunotoxicity is a major concern in drug safety evaluation. Drug-induced immune effects can result in a spectrum of changes from immunostimulation to immunodeficiency. Traditionally, histologic examination of H&E stained lymphoid organs has been used to identify drug-related effects on the immune system by characterizing alterations in the microscopic architecture of these organs. In addition, the pathologist may request immunohistochemical staining of various lymphoid subpopulations to assist with the microscopic evaluation and scoring. Regardless of the color that pathologists use to evaluate the tissue, their interpretation is still semi quantitative. A more quantitative assessment of lymphoid organs could improve the accuracy, sensitivity and speed of assessment. This work compares the two image analysis technologies for the quantitation of B-cell (CD79ac) and T-cell (CD3) area in rat lymphoid organs: 1) Definiens object-base image analysis utilizing 5x Nanozoomer virtual images of chromagen-stained sections and 2) laser-scanning cytometry (LSC) using fluorescent-stained sections evaluated at 20x. In addition, the results of both the Definiens and LSC analyses were compared to the subjective histologic score. A high-throughput methodology that is accurate and correlates with a pathologist's grading scheme will be a useful new tool for immunotoxicological evaluation.

METHODS

Male Sprague-Dawley (CrI:CD(SD)IGS BR) rats 6 to 8 weeks of age were treated with vehicle (saline) or cyclophosphamide (3 and 6 mg/kg) for 14 days. The immunosuppressive effects on T-cell dependent antibody responses were measured using the IgM response to KLH administered on day 10. Spleen, thymus and mesenteric lymph node were collected on day 15, fixed in 10% buffered formalin for histopathologic evaluation. Tissues were sectioned, stained with hematoxylin and eosin for standard morphologic assessment and stained for antibodies to B-cells (CD79a) and T-cells (CD3) for immunophenotyping. Slides evaluated by LSC were stained using fluorochromes with DAPI nuclear counterstaining and those evaluated by image analysis were stained using DAB with a hematoxylin counterstain. LSC evaluation consisted of using random phantom integrals (stereology) to determine the proportion of positive tissue area. A Definiens ruleset was developed to also identify the proportion of tissue area considered positive. Thresholds for both methods were set independently by the user with no effort to coordinate the results prior to comparison.

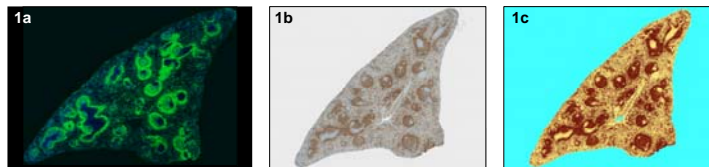


Figure 1. B-cell (CD79a) areas in a spleen: 1a) stained with fluorochromes AlexaFluor 488 (green) and DAPI (blue) for LSC analysis and (1b) stained with chromagens DAB (brown) with a hematoxylin counterstain. 1c) The results of a Definiens ruleset applied to image 1b are shown with positive staining B-cell areas colored brown and non-B-cell areas colored yellow.

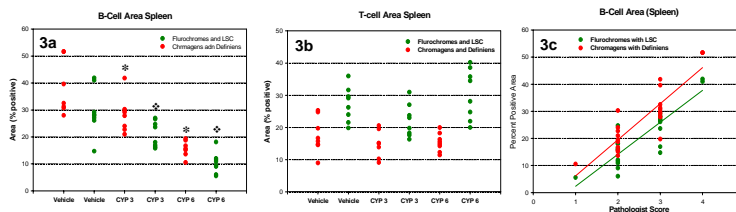
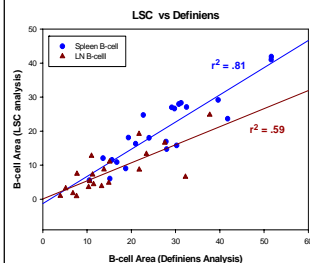


Figure 3. In spleens, the extent of B-cell area decreased significantly (p<0.05) in animals treated with cyclophosphamide 3 mg/kg/d (CYP 3) and 6 mg/kg/d (CYP 6) vs. treatment with vehicle. 3b) T-cell area was not altered. 3c) There was a good correlation between the measurement of B-cell area by laser scanning cytometry (r² = 0.75) and by Definiens analyses (r² = 0.81) with histopathology scoring of lymphoid area in chromogenic stained sections. Results of thymus B-cell and T-cell analyses are not presented because no treatment differences were found.

Comparison of LSC and Definiens Analysis



Spleen B-cell analyses correlate with r² = 0.81.

Lymph node B-cell analyses correlate with r² = 0.59.

The lower correlation for lymph nodes is likely due to greater variability in the morphology of lymph nodes than the spleen.

Less than perfect correlation between LSC and Definiens analysis may be due to several factors:

- 1: LSC measurement was done using a phantom integrals which is a stereological approach for estimating areas from tissue sections. In contrast, Definiens rule set measured the actual staining area of the entire tissue section.
- 2: LSC analysis was performed at 20x magnification while the Definiens rule set was performed on virtual 5x images.
- 3: Fluorescent and chromogenic stains may have differing sensitivity.
- 4: Analyses were on different tissue sections which may not be directly comparable.

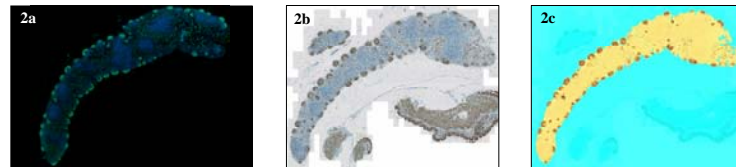


Figure 2. B-cell (CD79a) areas in a lymph node: 2a) stained with fluorochromes AlexaFluor 488 and DAPI for LSC analysis and 2b) stained with chromagens DAB and hematoxylin for Definiens analysis. 2c) The results of a Definiens ruleset applied to image 2b are shown with positive staining B-cell areas colored brown and non-B-cell areas colored yellow.

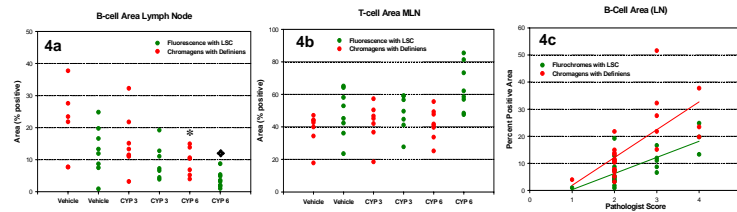


Figure 4. In lymph nodes, B-cell area decreased significantly (p<0.05) in animals treated with cyclophosphamide 6 mg/kg/d (CYP 6) but not 3 mg/kg/d (CYP 3) vs. treatment with vehicle. 4b) T-cell area was not altered to a statistically significant extent. 4c) The correlation between the measurement of B-cell area by LSC (r² = 0.52) or Definiens analysis (r² = 0.66) and histopathology scoring of lymphoid depletion in chromagenic stained sections was less than the correlations obtained with analysis of spleen.

CONCLUSIONS

Image analysis of virtual images using Definiens eCognition software can be used to quantitate T- and B-cell populations in rat lymphoid tissues.

These data were positively correlated with LSC data, serologic evidence of decreased IgM and semiquantitative assessment by a pathologist.

The lower correlation for lymph nodes is likely due to greater variability in the morphology of this organ than the spleen.

Differences in the correlation coefficients between virtual 5x chromagen-stained sections analyzed via Definiens technology and 20x fluorescently-stained sections analyzed via LSC were most likely due to the object-based quantitation used by Definiens versus the stereological quantitation used by LSC.

The results of this study defined that treatment with cyclophosphamide significantly decreased B-cells but not T-cells in the spleen and mesenteric lymph nodes.

Quantitative analysis of other lymphoid cell populations and subsets should be possible.