1995

Flow Cytometric Analysis of Avian Blood Cells: Differentiation of Erythrocytes and Leukocytes by Fluorescence.

William Weaver King
Louisiana State University and Agricultural & Mechanical College

FLOW CYTOMETRIC ANALYSIS OF AVIAN BLOOD CELLS: DIFFERENTIATION OF ERYTHROCYTES AND LEUKOCYTES BY FLUORESCENCE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences
through the Department of
Veterinary Pathology

by
William W. King
B.S., Rhodes College, 1987
D.V.M., Louisiana State University, 1991
August, 1995

Acknowledgments

There have been so many individuals involved in shaping this work that mentioning them all would require volumes. However, I must express my appreciation to Dr. Stephen D. Gaunt, who accepted the role of my graduate advisor somewhat unexpectedly with never a lack of enthusiasm or encouragement. His leadership, along with the intuition and optimism of Dr. Sharon M. Dial, formed the foundation for these studies. In addition, the other members of my graduate committee, Dr. H. Wayne Taylor, Dr. Julian L. Oliver, and Dr. Domonique G. Homberger provided assistance and advice "above and beyond."

I cannot help but single out the remaining committee member, Dr. W. Sheldon Bivin. Dr. Bivin's guidance throughout the inception and development of my Veterinary profession career reflects not only his abilities as a mentor, but also his sensitivity and benevolence.

Without the untiring assistance of several other people, this work would have been impossible. Marilyn A. Dietrich of the Flow Cytometry Facility provided expertise and service that cannot be overstated. Mae K. Lopez of the Department of Veterinary Pathology contributed significantly to the immunological experiments. The technical support of Del Phillips and Cindy M. Berry of the Department of Veterinary Pathology is also noteworthy.

It is difficult to express the professional satisfaction yet emotional turmoil this treatise represents. Although certainly a task better viewed upon completion, the culmination of this work also signifies the ending of my education at Louisiana State

University. I must voice my gratitude to the entire faculty and staff of the Louisiana State University School of Veterinary Medicine. In the past eight years, this exemplary collection of individuals has served as instructors, counselors, peers, and friends.

A supportive family is often mentioned. In this case, their role has never been regarded more sincerely. I credit my mother, father, and sisters for giving me the conviction and perseverance to fulfill all of my educational successes. My greatest expression of appreciation, however, goes to my wife, Catherine. Her tireless faith and perpetual assurance has given me the ability to overcome otherwise insurmountable obstacles. Her confidence in me has resulted in a recent event eclipsing all other accomplishments, the birth of our son, Douglas.

These studies were supported by grant from the American Federation of Aviculture. The assistance of Dr. F. J. Dein for preliminary studies is especially notable.

Table of Contents

List of Tables

List of Figures

Abstract

Automated analyzers have revolutionized diagnostic hematology in mammalian species. These commercial instruments utilize flow cytometric technology to enumerate blood cell concentrations. Because of the nuclei present in non-mammalian erythrocytes and thrombocytes, these instruments are unable to calculate leukocyte counts in birds, amphibians, reptiles, and fish. These investigations sought to determine if three commonly used methodologies in flow cytometry could sufficiently differentiate avian erythrocytes, leukocytes, and thrombocytes, and ultimately form a basis for performing total leukocyte counts.

Fluorescein isothiocyanate (FITC) and thiazole orange (TO) were used to stain samples of whole, erythrocyte-, and leukocyte-enriched chicken blood. Although fluorescent microscopic and flow cytometric results obtained using both stains suggested a higher propensity for these dyes in leukocytes and thrombocytes, the difference in fluorescence intensity with erythrocytes was not sufficient to assess their concentration. Furthermore, leukocytes stained with FITC were found consistently in the large erythrocyte peak in cell sorting experiments. Cell counts performed on a population of cells defined by higher TO staining correlated poorly with manual total leukocyte counts.

Chicken blood cells were also examined for reactivity with anti-spectrin, anti-vimentin, and anti-β-tubulin antibodies. Leukocytes demonstrated a higher non-specific staining with secondary antibodies. The inclusion of normal serum as a

blocking step essentially eliminated this reactivity. The non-specific staining was not detected by flow cytometry.

Although these investigations verified that standard flow cytometric techniques may be utilized to analyze avian leukocytes, sufficient differentiation of these cells from erythrocytes was not achievable for quantitative purposes. Methods with increased sensitivity of fluorescence detection or improved specificity of leukocyte staining are needed to develop a system by which this important diagnostic evaluation can be automated in non-mammalian hematology.

Chapter 1: Introduction and Literature Review

Companion avian and other non-mammalian exotic animal medicine has profoundly impacted the veterinary health care market. A 1984 census of 13,506 households in the United States by Charles, Charles, and Associates, Inc., revealed that 5.0% of American homes contained at least one bird. This study also divulged that 7.3% owned fish and 0.5% owned reptiles (1). By 1987, the reported number of pet birds rose to 5.7%, with a mean number of birds per bird-owning household of 2.5 (2). As the companion exotic and avian market continues to expand, so grows the demand for accurate and precise clinical diagnostics.

The proper diagnosis and subsequent treatment of disease is of particular gravity for endangered species. Heightened public awareness of the critical role of many non-mammalian wildlife species necessitates innovative improvements in diagnostic capabilities. Additionally, federal agencies such as the National Institutes of Health continue to investigate the development of non-mammalian research, especially as it pertains to models of human disease. With increased use of avian, amphibian, reptilian, and fish species in the laboratory follows the need for reliable hematological analysis.

A complete blood count including a total leukocyte count is an essential component of the diagnostic minimum data base in all mammalian species; its importance in non-mammals is no less. Numerous systemic diseases result in elevation or depression of the leukocyte concentration (3). Unfortunately, the current

methods of determining total leukocyte counts in these species rely on manual laboratory techniques.

The advent of flow cytometry as a diagnostic tool permits scrutiny of individual cells in heterogeneous samples. With this technology, scientists are able to survey subpopulations of cell suspensions while simultaneously inspecting or ignoring others. Fluorescent dyes or labeled immunoglobulins allow the identification of unique aspects of single cells or cell classes. These works sought to evaluate flow cytometric techniques and their ability to differentiate chicken erythrocytes and leukocytes, and thus form a basis for calculating a more rapid and accurate total leukocyte count.

Overview of Flow Cytometry

Flow cytometry (FCM) has greatly facilitated the rapid evaluation of physical and biochemical properties of individual cells in suspension (4). The successes of FCM are partially due to advances in monoclonal antibody and fluorescent dye technology and their integration with computer-driven cytometric instrumentation (5). Flow cytometric methods are now employed in a growing number of research and clinical settings. Flow cytometry has been used to analyze numerous biological bodies, ranging from immune complexes and viruses to neoplastic cellular clumps and small multicellular organisms (6).

Basically, a flow cytometer consists of a system of fluidics which focus a suspension of cells into a single stream. This stream flows through an interrogation point, where cells pass through a light beam, usually provided by a laser. Signals

produced by this interrogation, such as light scatter, fluorescence, or absorbance, are analyzed by computer interface (4). Since cells are evaluated individually, properties such as size, density, and dye uptake can be evaluated on a particular population of cells within a heterogeneous sample (7). Using pre-established criteria, electrical fields can be generated around a particular cell as it exits the interrogation point and diverted into collection vessels. This process of cell sorting can be performed for any measurable parameter (8).

There are many advantages of FCM in diagnostic pathology when compared to other cytometric methods. These include the rapid analysis of up to 10,000 cells per second, sensitivity to as few as 2000 molecules of a fluorochrome, simplicity in sample preparation using small volumes, and the ability to examine single cells in heterogeneous samples (5,9). One of the few disadvantages to the use of FCM involves sample preparation. Solid tissues require dispersion by physical and enzymatic reactions which often increases the amount of cell damage and debris (4). The most severe limitation for many investigations is the cost of purchasing, maintaining, and operating the FCM units (10). However, as with most diagnostic instrumentation, the trend to decrease both the complexity of operation as well as the purchase price will likely continue (5,9).

History

In Practical Flow Cytometry, H. M. Shapiro provides an excellent account of the early history of FCM (11). He credits Moldavan as the originator by attempting to count cells suspended in a capillary tube by photo-electric means (12). Instruments

which used filtered air as a sheath stream and a Ford headlamp as a light source to analyze aerosol particles in mine dust and airborne microorganisms were described in the late 1940's (13-15). Pioneering studies of the difference in DNA, protein content, and metabolism in normal and abnormal cell growth were described by Caspersson using ultraviolet and visible light absorption in photographic microspectrophotometry (16).

In 1956, W. H. Coulter created the first Coulter-Counter. This instrument not only counted erythrocytes but also measured their size by diluting blood cells in saline medium and detecting the variations in electrical conductivity caused by the cells passing through a small orifice (17). This technology is utilized in many FCM instruments, including a number of hematology units currently manufactured (18-32).

In studies designed to measure DNA content in neoplastic cells, Kamentsky recognized the need to examine multiple parameters simultaneously, and designed an instrument in the 1960's with the optical and statistical acumen to measure both light absorption and scatter (33). Also at this time, cell sorters were produced, with the principal function to allow verification of FCM analysis by visual examination of the sorted cells (34,35). The Cytograf and Cytofluorograf, produced by Kamentsky, became the first FCM units to be commercially available for research purposes (36). Becton-Dickinson later produced a machine using a powerful argon ion laser which was able to discern the faint fluorescence of fluorescein and rhodamine labeled antibodies (36).

Early FCM instruments were restricted to selected research institutions, and, for the most part, designed to perform limited analyses such as comparison of cell morphology, delineation of lymphocyte subpopulations, and inspection of cell kinetics (37). It was not until the 1980's that units could perform multiparameter analysis, such as detection of multiple scattering angles and fluorescence (37).

Technological overview

Fluidics systems

Analysis of individual cells in suspension is made possible by a system of fluidics which operate under laminar flow conditions (4). Cells suspended in a liquid medium, or "core" fluid, are introduced through a narrow opening at a rate of approximately 1 mm/sec coaxially into a larger, rapidly-moving, cell-free, sheath fluid (usually about 10 m/sec) (9,38). This results in a hydrodynamic focusing of the cells in the sample fluid which then pass through the observation or "interrogation" point (5). Many different sheath fluids have been utilized. Isotonic saline is probably the most common, and due to its electroconductivity, is necessary for Coulter volume calculation (39).

Signal production

Most current FCM units utilize a laser light source for illumination. Lasers provide powerful, monochromatic beams that can be focused nearly to the dimensions of a cell and adjusted to produce several suitable wavelengths (5). Several have been employed in FCM systems, including helium-neon, helium-cadmium, krypton, and CW (continuous wave) dye lasers (40). The most popular is the argon ion laser, which

provides wavelengths of excitation useful for popular fluorochromes such as acridine orange, thiazole orange, ethidium bromide, propidium iodide, fluorescein, rhodamine dyes, cyanine dyes, and pyronin Y. Other light sources, such as the mercury short arc, xenon, and quartz-halogen lamps (40), are less expensive to purchase and maintain, but are not as adaptable (5). Fluorescent dyes bound to cellular structures such as DNA, RNA, or cytoplasmic protein, as well as to antigen-specific immunoglobulins, are readily excited by the wavelengths produced by laser illumination.

When illuminated, these dyes emit light of lower energy and therefore longer wavelength than the incident light, which is then collected by optical detectors (5,9). Optical filters placed in the path of the emitted light further discriminate background fluorescence and increase sensitivity (41). Numerous fluorochromes, or metachromatic chemicals which emit several different wavelengths, may be used in conjunction with multiple detectors (5).

"Orthogonal geometry" relates the perpendicular placement of the optical detectors in relation to the course of the illumination beam and the sample stream (42). With this arrangement, measurements of light scattered by a cell in the observation point are attained. Light scattering is a complex phenomenon utilized in most FCM instruments. Its analysis resembles dark field microscopy, since the identification of cellular characteristics are based on reflected rather than transmitted light (43). The intensity of illumination provided by lasers facilitates this examination.

The amount of light scattered depends on several characteristics of cell morphology, primarily cell size and cytoplasmic contents (42). The light bypassing a

cell in the observation point, referred to as forward scatter (FSC), can be proportional to the size or volume of the cell (9). This is collected by optical detectors between 2° and 13° relative to the laser beam (9). Side scatter (SSC), or reflected light, is measured at various higher angles from the laser beam. Side scatter can result from cellular constituents and has been correlated to cell granularity (5).

Signal detection

Collection of information from the interrogation point depends primarily on the type of signal produced. The Coulter principle relates cell characteristics to the presence and magnitude of impedance displacement across electrodes surrounding a small orifice. This produces a voltage spike which can be quantified and transmitted electronically (17). Many FCM units are restricted to light signals. The collection of light signals is based essentially on light microscope optics. Lenses, prisms, and filters are arranged such that large amounts of light are retrieved from the observation point, with relatively small amounts from the surrounding areas (40). Dichroic reflectors limit transmission to wavelengths only less than a given value; band-pass filters transmit wavelengths only in a given range. These optical filters provide specificity of wavelength detection (9).

Detection is consistent in most commercially available FCM units. Photodiodes convert optic signals into electronic impulses similar to solar cells (5). Fluorescence is detected by photomultiplier tubes, which also create a current pulse when struck by light of the appropriate wavelength (44). These analog impulses are translated into digital signals (numerical values) by analog-to-digital converters (5).

Instrument operators can control the sensitivity of the optical detectors for each available parameter. Adjustment of these photodiode and photomultiplier settings alters the appearance of the data significantly when the information is displayed (see "Data Analysis").

The alignment of the illumination beam to the cell stream and detectors is crucial, most importantly for scatter measurements. Minor alterations in the position of individual cells profoundly affects the amount of light that is perceived as scatter (43).

Cell sorting

One of the more powerful tools available in FCM instruments is the cell sorter. This feature allows not only the collection of data from each cell, but also the physical separation of populations into specified vessels. Cells of interest are confined to individual droplets of the sheath fluid, formed beyond the interrogation point by vibrating the stream with a piezoelectric crystal oscillating the sample nozzle at a high frequency, usually between 20-40 kHz (5,9). These droplets are then charged, either negatively or positively, and displaced from the main stream by passing through an electromagnetic field (9). Since the course of the residual, uncharged droplets is unaltered, three different populations of either mixed or pure cells can be sorted and further analyzed (45).

Any parameter or set of parameters that distinguishes an individual population of cells can be used for sorting (4). Thus, cell types which are found in low numbers in a heterogeneous sample may be purified (9). The amount of purity attainable

depends only upon the sensitivity and specificity of measuring the specified parameter(s) (45).

Cellular parameters

Shapiro classifies parameters that investigators have used to study cells via FCM in two mutually exclusive categories: intrinsic and extrinsic, structural and functional (37). Intrinsic parameters are those that do not require a reagent to analyze; conversely, extrinsic parameters require the addition of a reagent. The distinction between functional and structural is somewhat nebulous, as both are interdependent. Functional parameters are those processes and reactions that may be identified by changes in their respective states. Structural parameters concern inherent physical aspects, such as granules, antigens, and cytoskeletons. Examples of each parameter include cell size, shape, granularity, and autofluorescence for intrinsic structural; oxidation-reduction state for intrinsic functional; nucleic acid and other macromolecule content and surface antigens for extrinsic structural; and membrane state, enzyme function, and ion content for extrinsic functional parameters. Several of these parameters have been utilized in automated hematology instrumentation, including the structural intrinsic parameters of cell size and granularity, and various extrinsic parameters such as enzyme activity.

Cell size and forward scatter

Some of the principles of cell size measurement have been discussed. Perhaps the most commonly used method is the Coulter volume, which measures the magnitude of impedance change occurring when a cell passes through an aperture

across which is a steady voltage (17). Cell size is measured optically by forward scatter (FSC), between 2° and 13° (9). The correlation between FSC and cell size or volume is based on the assumption that the cell examined resembles a homogeneous sphere, and may not hold true for non-spheroid cells (5,46,47). Many other factors influence the FSC measurement. These include variations in refractive index of the cell and surrounding fluid, cellular contents, light absorption by cellular elements, and the wavelength of the illuminating light (48-50). Therefore, caution must be exercised when comparing FSC-based cell size in two different populations of cells, especially if the cell types differ in internal structure (37). For example, human granulocytes are often perceived as smaller than lymphocytes when UV light is utilized (43).

Regardless of its limitations, many hematological instruments use FSC as an estimate of cell volume to differentiate populations of blood cells. To compensate for aberrant findings using FSC in non-spherical cells, such as erythrocytes, some FCM units physically transform the cells into spheres (51). Another method of measuring cell volume is slit-scan or "time of flight" analysis, which calculates the time required for a cell to pass through an interrogation beam in which at least one axis is significantly smaller than the shortest diameter of the cell. The pulse width produced is comparable to cell size (48,52).

Other uses of FSC have been described. Cell contents that absorb illuminating light interferes with FSC; this has been utilized to classify cells using extinction measurements (37). An early instrument built by Curbelo *et al.* distinguished

erythrocytes and leukocytes by the absorption of certain wavelengths of light by hemoglobin, although a laser-driven unit created later provided poorer results (53,54).

Cell contents and side scatter

Internal cellular structures provide opportunities for scattered light over a wide range of angles from the incident beam. This "side scatter" (SSC) has been used as a measure of cytoplasmic granularity (5,55). For this evaluation, most FCM instruments typically contain detectors at right angles to the incident beam. The SSC has also been used to estimate total cytoplasmic protein content (37). The interpretation of SSC may be confounded by sample preparation techniques, such as fixation and permeabilization (55).

Units that use arc lamps as light sources furnish another method of differentiating cells by granularity (55). These measurements of light scattered over a large range of wavelengths, aptly termed "multiple wavelength scattering," were incorporated in several early hematology units. Samples such as whole blood can be differentially dyed so that granulocytes can be identified by absorbing and scattering unlike wavelengths. The ratio of absorption and reflection can also be used to discriminate populations.

Fluorescence

Autofluorescence of cellular compounds has been utilized as an intrinsic parameter. Autofluorescence is thought to be due primarily to the presence of flavin and pyridine nucleotides which emit light in the blue and UV wavelengths (56,57). The fluorescence of such coenzymes has been used to appraise metabolic (red-ox)

state (58). Many reports, however, refer to this autofluorescence as an interference with extrinsic fluorescence measurements (59).

Quenching of heme by the iron moiety in hemoglobin renders the molecule non-fluorescent. However, certain manipulation steps result in hemoglobin fluorescence. This phenomenon was utilized to evaluate erythrocytes by excitation in the violet wavelengths (406-422 nm), which result in orange-red fluorescence (59).

Extrinsic fluorescence is usually provided by fluorescent dyes, or fluorochromes. Two major mechanisms by which fluorescent chemicals distinguish cell components have been described (60). The first is by establishing appreciable contrast with a non-fluorescent background, which can be provided with two dyes with disparate fluorescence capabilities and affinities for different cellular components. Secondly, certain fluorochromes have increased quantum efficiency when bound to a substrate (e.g. DNA) or exposed to certain conditions (e.g. altered pH), and therefore increased fluorescence.

A frequently performed flow cytometric assay is the determination of nucleic acid content utilizing intercalating dyes such as ethidium bromide, propidium iodide, and acridine orange. These fluorochromes exhibit increased fluorescence upon binding to DNA and RNA, thus providing sufficient contrast to the unbound dye in the surrounding medium (60). Acid dyes such as fluorescein isothiocyanate (FITC), which non-specifically binds to proteins, must be washed from the medium to reduce

background interference since fluorescence intensity is not increased when bound (60).

Another popular use of FCM is the classification of cells based on the presence of distinguishing antigens identified by fluorochrome-labeled antibodies. The most commonly employed fluorochrome is FITC (37). The excitation range for FITC between 460-510 nm, with an green emission range of 500-550 nm, correlates well with the argon ion laser used in most FCM with maximal illumination of 488 nm (61). Fluorescein has also been used to label isolated hormones and other ligands to identify their corresponding cytoplasmic receptors (62,63).

Data analysis

Optical signals are generated at the observation point, translated into electric pulses, amplified, converted to digital signals, then stored and displayed with the assistance of electronic components and computers (4,5). Each signal falls into an intensity range known as a "channel," which has a corresponding numerical value. Resolution is therefore determined by the number of channels used to collect the information, usually 1024 (5). Values for each parameter are collected and stored for every cell, and can be analyzed independently or simultaneously.

Visualization of a single parameter, such as FSC or single-wavelength fluorescence, is generally accomplished with frequency histograms (4). Considerable overlap of different populations often occurs with single parameter analysis. Examination of two or more parameters simultaneously, using dot, contour, or chromatic plots, usually delineates separate populations (64). In addition, many

instruments allow a choice between a linear or logarithmic scale for axis labels. Parameters that follow a stoichiometric model, such as cell size and DNA content, are best analyzed using a linear amplification (9). Logarithmic amplification permits examination of two populations which differ greatly on the same diagram (43).

In dot plots, or scatter diagrams, numerical or channel values for each parameter are represented in two dimensions by points or dots for each cell examined. Frequencies are indicated by clustering; white areas on screens, or black areas on paper, indicate higher frequencies (64). Contour plots contain a series of contour lines, or isopleths, which connect data points having identical frequencies. Chromatic plots are created using the same concept, with the planes of contour lines replaced by different colors or shades. Frequency in addition to two parameters can be displayed in a three-dimensional histogram, in which a fabricated surface depicts higher frequencies as peaks (64). While these three-dimensional plots may be perplexing to the inexperienced, small populations that are otherwise obscured may become apparent (43).

The production of two-dimensional plots allows the use of "gates," which are user-defined boundaries drawn around populations or areas of interest. Not only can quantitative information be calculated, but additional analysis may also be performed exclusively on cells falling into predetermined gates. For example, a small population as defined by FSC vs. SSC can be circumscribed, and the fluorescence measurements examined solely for these cells rather than the entire sample.

A significant amount of information must be processed for each cell analyzed, resulting in massive data files. Computer-based support is necessary, as 4-6 parameters on tens of thousands of cells are often collected (10). Commercial units are usually supplied with standard analysis software. In addition, several manufacturers provide specialized programs for maneuvering data. For example, "Paint-a-Gate" (Becton-Dickinson Immunocytometry Systems, San Jose, CA) allows the user to color certain populations from a dot-plot or histogram and follow the painted events through displays of any other parameters (9).

Applications

Several texts provide comprehensive overviews of applications for flow cytometric technology and techniques in research and clinical endeavors (11,65). These include the fields of cell biology, genetics, immunology, microbiology, parasitology, and reproduction in research investigations; and immunology, oncology, microbiology, and hematology in clinical settings. The ability of FCM to characterize and analyze surface antigens has essentially revolutionized the fields of immunology and cell biology (4). Of particular interest, the first clinical use of flow cytometric technology was in hematology with the Coulter cell counter (17). With the advent of erythrocyte lysing solutions, leukocyte counts were also possible. These instruments offered considerable savings in time, effort, and reproducibility in comparison to manual methods (19,66).

Automated Leukocyte Counts

Preliminary considerations

Historically, erythrocyte, leukocyte, and platelet counts were performed by manual methods utilizing the hemacytometer, a scored slide with a chamber which holds a specified volume of diluted blood (67). This execution is plagued with imprecision and inaccuracy, due primarily to the error inherent with small sample sizes. Significant populations of rare cells can be easily missed, and biological variation magnified, with the small amount of blood examined (68). Although automated counters may risk an error rate of 5%, manual methods often experience over 20% (17,69).

Early FCM studies demonstrated their potential in analytical hematology. Coulter instruments were originally designed for erythrocyte studies (17), and since that time many instruments utilizing numerous methods have been devised for blood cell analysis.

Leukocytes have been subjected to many investigations. Flow cytometry has facilitated description of functional parameters in neutrophils such as membrane potential changes, oxidation state, degranulation, and phagocytosis (10,70). Bare nuclei of inflammatory cells have been identified in tissue samples using FSC and SSC alone (10). Both FSC and SSC measurements in conjunction with cytochemical reactions have been used to differentiate leukocytes in peripheral blood (37,71,72). Flow cytometric differential leukocyte counts were also performed using the metachromatic dye acridine orange, which distinguishes DNA and RNA content by

emitted different wavelengths (73). Immunofluorescence markers are frequently exploited to identify and classify lymphoid and myeloid cells (10). Generally, hematologic instruments lyse erythrocytes prior to leukocyte analysis, however, these cells have been differentiated by the absorption of certain wavelengths by hemoglobin (53,72).

Because of the augmentation in precision, automated instrumentation has essentially replaced manual methods (67). For example, counts of at least 2500 cells are necessary to attain a coefficient of variation (CV) of 2% (74). Automated instruments frequently examine over 10,000 cells, resulting in vast improvements in reproducibility. Kjeldsberg tabulated comparisons of the difference in CV between erythrocyte (11% vs. 1.0%), leukocyte (16% vs. 2.0%), and platelet (22% vs. 4.0%) counts in hemacytometer and electronic methods, respectively (67).

Automated methods have additional advantages. The graphic displays not only permit better identification of aberrant populations, but also foster the future identification of abnormalities unrecognizable by light microscopy (75). Reduced technical time and training may render automated methods more cost effective (19,68). An interesting limitation of early instrument-generated cell counts was their reliance on manual methods for calibration, although many manufacturers now provide calibration standards (76).

The approximately 45 commercial units that are now manufactured for human hematology utilize either electrical impedance or optical methods (67).

Impedance instruments

Several instruments use electrical impedance to estimate cell size and concentration, including those manufactured by Coulter (Coulter Electronics, Hialeah, FL) (21,22,77), Sysmex (TOA Medical Electronics, Carson, CA) (78), Serono Baker (Baker Instruments Corporation, Allentown, PA) (77,79), and Clay Adams (Parsippany, NJ) (23,24,67). Both erythrocytes and platelets are distinguished by gating of volume differences, although platelet counts may also be performed in platelet-enriched plasma (23,24,78,80). Leukocytes are usually included in the erythrocyte analysis. Their relatively low numbers create significant artificial increases in the erythrocyte count only if the leukocytes concentration is greater than 50,000/μL (74). For leukocyte enumeration, samples are diluted in a reagent that lyses the erythrocytes and shrinks the leukocytes to 10-20% original volume (32).

Some units, such as the Coulter S-plus series, STKR (20,22,81), and Sysmex E-5000 (26,27,81), calculate a partial leukocyte differential based on measured volume differences: lymphocytes between 30-90 fL, mononuclear cells between 90-160 fL, and granulocytes between 160-450 fL (20,67,68). These somewhat arbitrary ranges are automatically adjusted to compensate for individual patient variation, referred to as "floating thresholds" (22).

Several of these impedance instruments have been investigated for use in mammalian veterinary species and found to function satisfactorily, although most require modification (3,30-32,82). In controlled studies, the Coulter S-Senior and S-Plus, with slight electronic adjustments such as aperture current and threshold settings,

were both expected to perform satisfactorily for dogs, cats, horses, and cows (30). The Coulter Model S550 also performed well with slight modification of aperture currents for these species (31). Other species present various problems. For example, the S-Plus IV often calculated inaccurate leukocyte counts in both feline and bovine samples, although reducing the aperture voltage improved correlation with previously accepted methods (82). The Sysmex E-5000 was been found to be inappropriate for veterinary use without significant modification (82).

Several hematology instruments manufactured specifically for the veterinary market utilize electrical impedance, including the Mascot Multispecies Hematology systems (CDC Technologies, Inc., Oxford, CT) and the Danam Vet 8/HD-11 and Vet 5/HD-11 Hematology systems (Danam Electronics, Inc., Dallas, TX). Both of these units employ lysing agents to remove erythrocytes when calculating the leukocyte count. In addition, the Mascot performs a partial leukocyte differential based on volume histograms (83). In a recent survey, Gaunt *et al.* reported frequent use of the Coulter S Plus IV and Serono Baker 9000 in veterinary teaching hospitals and private diagnostic laboratories (77). The Serono Baker 9000 and Ortho ELT-8/ds, an optical instrument, demonstrated comparable results using blood from dogs, rats, and mice (79).

Optical instruments

Optical hematology units, including the Ortho ELT counters (Becton-Dickinson Co., Rutherford, NJ) (79,80,82,84), Technicon Hemalog-D (19,28), H-6000 (85) and H-1 (75,82,86-89) (Technicon Instruments Corporation, Miles, Inc.,

Tarrytown, NY), and Celldyn hematology analyzers (Abbott Laboratories, Abbott Park, IL) (81,90) use strong light sources, usually lasers, to illuminate individual blood cells in a focused stream. This illumination generates several measurements, including forward scatter, side scatter, and other assessments specific for these instruments (91).

The Ortho units count and differentiate erythrocytes from platelets by calculated volume, refractory index, and "time of flight" measurements (67). Leukocytes are identified based on size following partial lyses (shrinkage) and light scattering, producing a three-part differential similar to impedance counters (92). In addition to the Serono Baker 9000, the Ortho ELT-8/ds and ELT 8/ws were independently tested and compared with the acceptable results from a Coulter Model S-Senior system in veterinary species (82). Both of these units performed well, although many erythrocyte parameters and feline leukocyte counts were inaccurate. Although the three-part differential counts available on the Ortho units did not correlate well with manual counts, the authors commented that these instruments may serve effectively as a screening tool (82).

Technicon has produced a number of hematology machines based on FCM principles and optics. Erythrocyte and platelet concentration and volume are calculated using forward scatter measurements (89). Following erythrolysis, leukocytes are counted and differentiated by various mechanisms, including cytochemical reactions for peroxidase activity. This parameter is measured by adding a chromogen and hydrogen peroxide, then quantified by bright field detectors for

absorbance. The Hemalog-D, the first commercially available flow system to provide a differential count for human blood, distinguished leukocytes based on both cell size and cytochemical profiles: neutrophils and eosinophils via peroxidase, monocytes via nonspecific esterase, basophils by Alcian Blue, and lymphocytes by size and absence of other staining (19,28). The H-6000 uses only cell size and peroxidase activity to differentiate the leukocyte series (85,89). The H-1 uses two light sources, a tungsten-halogen for peroxidase measurements as in other units, and a helium-neon laser for erythrocytes, platelets, and basophils (75,82,86-88,93). A special reagent "isovolumetrically" spheres and fixes the erythrocytes, so that forward scatter measurements of mean corpuscular volume are more accurate. A detergent lyses all cells except basophils; forward scatter identifies these cells, while side scatter measurements are used to estimate nuclear "lobularity." This provides an additional method of differentiating granulocyte (neutrophils and eosinophils) and mononuclear (lymphocytes and monocytes) nuclei for comparison with the results of the peroxidase channel.

The Technicon H-1 was evaluated for use in veterinary species and found to perform extremely well for canine and equine samples when compared to the Coulter S-Senior system (82). Software designed for various veterinary species accompanies the newer, H* system (75). Differential counts correlated fairly well for canine blood (82), and have since been substantiated in several other species (75).

The Celldyn systems utilize a "Multi-angle Polarized Scatter Separation" system to identify and classify leukocytes after a reagent modifies erythrocytes to

minimize their scatter (90). Leukocytes are characterized by a combination of four parameters: cell size by forward scatter (low-angle, 1-3°); complexity by wide angle scatter (3-11°); lobularity by orthogonal scatter (90°), and granularity by depolarized orthogonal scatter (81,90).

Although a number of instruments are currently manufactured, the overwhelming majority are designed to analyze blood from a single mammalian species -- human. While several of these machines can be successfully modified for veterinary applications, no automated instrument has been validated for non-mammalian blood. In all of these systems, the total leukocyte count is simplified by lysing the erythrocytes and enumerating the remaining shrunken leukocytes or their nuclei. The presence of nuclei in the erythrocytes and thrombocytes in non-mammalian blood results in aberrant classification of all cells as leukocytes in these machines, i.e. there is a lack of differentiation between erythrocytes and leukocytes. There is considerable interest in adapting available units for use in birds. Some commercial laboratories use automated systems for erythrocyte counts (94). Additionally, total leukocyte trials with the Celldyn are underway in certain diagnostic laboratories (77). However, confirmation of this and other potential instruments remains untested in these species.

Hematologic Counts in Non-mammalian Species

Manual methods

There are three methods of determining the total leukocyte count in avian and other non-mammalian blood samples: direct, semi-indirect, and indirect. The

simplest is the indirect method, whereby the ratio of leukocytes to erythrocytes is determined on a stained blood smear. The leukocyte count is then calculated by comparison to the absolute erythrocyte count achieved by a hemocytometer (95). Another indirect leukocyte estimate commonly used averages the number of leukocytes per high dry (40x) field multiplied by 2000 (94). These methods have several disadvantages. They rely on a blood smear with a homogeneous distribution of cells, a packed cell volume within normal limits, and the ability of the observer to distinguish the leukocytes present (96). Although a technologist may become quite precise in time, the degree of accuracy attainable is questionable.

The direct methods currently available utilize one of three solutions to preferentially stain leukocytes, permitting the technologist to count individual cells in a hemacytometer. These stains include Natt and Herrick's (97), Shaw's (98), and the Modified Rees - Ecker solution (99,100). The disadvantages in these techniques are the time required to count statistically significant numbers of cells, and the difficulty in distinguishing leukocytes from thrombocytes and erythrocyte precursors, which can inflate the calculated leukocyte count (101). Additionally, these stains are user-prepared, often unstable, and laborious to use (102).

The semi-indirect method described by Wiseman (103) is one of the more popular methods of determining total leukocyte counts (77). It exploits the fact that heterophils and eosinophils absorb phloxine dye. The Unopette® system for human eosinophil enumeration (Test 5877, Becton-Dickinson, Vacutainer Division, Rutherford, NY) utilizes this property by measuring a given quantity of whole blood

and staining the appropriate cells in a convenient and disposable delivery system. Stained blood is placed in a hemacytometer and the visible cells are counted, yielding an absolute heterophil/eosinophil count. The total leukocyte count is then ascertained by comparison to the differential blood count performed on a standard blood smear.

Many limitations have also been identified with this method. Positively-stained cells are often not easily discernable from cellular debris and erythrocyte precursors. There is a high degree of precision if both the counted cells and the differential count is high, but this precision is greatly reduced if either is low (104). In addition, an error margin of 32% has been reported when using any hemacytometer reading (105).

Perhaps the major disadvantage lies in the fact that the semi-indirect system relies on an accurate differential count. This introduces a great amount of subjectivity due to technologist experience and/or bias. A given margin of error may be insignificant when interpreting the differential, but have profound effects on the calculated leukocyte concentration (96,102). Furthermore, there remains significant controversy as to the authenticity of avian leukocyte identification. Many of the leukocytes are difficult to distinguish, especially thrombocytes from small lymphocytes (106). Incorrect identification may deviate the total leukocyte count by altering the relative amounts of heterophils/eosinophils and lymphocytes.

Electronic methods offer the highest degree of precision and accuracy in performing blood cell counts. With automated systems, the extended time period required to train technologists in manual methodology is unnecessary (94). A

conservative average of 10 - 20 minutes per sample to properly perform manual counts could be reduced to a few minutes for several samples. By developing a system of distinguishing leukocytes from nucleated erythrocytes, the enigma of automated hematology in non-mammalian species could be resolved.

Adaptation of automated methods

Many different parameters have been examined to distinguish leukocytes from other blood cells by electronic cell counters. These include cell size and granularity, cytochemistry, fluorescent dyes, autofluorescence, absorption, or combinations thereof. Many of these methodologies may be adapted to avian blood, but the majority are likely inapplicable to species with nucleated erythrocytes and thrombocytes.

Cell size

Most hematology instruments classify mammalian leukocytes based solely on intrinsic characteristics, with cell size the most frequently used parameter. Many instruments measure cell volume by electrical impedance (20,22,26,27,67,81). After lysing erythrocytes, shrunken leukocytes remain to be enumerated and differentiated by size into a three-part differential of lymphocytes, monocytes, and granulocytes. Optic-based hematology machines perform leukocyte identification based on both FSC and SSC as indicators of differences in cell size and internal structure (67,92,107). Some instruments also use light scattered over several wavelengths to aid in categorization (55,81,90).

It is not possible to differentiate avian erythrocytes, thrombocytes, and leukocytes based on volumetric measurements alone. According to Lucas and Jamroz (108), the average dimensions for thrombocytes and erythrocytes is 4.7 x 8.5 μm and 6.8 x 11.4 μm, respectively. The average diameter of avian leukocytes: 7.7 μm (lymphocytes), 12.0 μm (monocytes), 8.7 μm (heterophils), 7.4 μm (eosinophils), and 8.2 μm (basophils), falls directly within these ranges. Thus, unless reagents were used to alter the volume or size of erythrocytes and leukocytes differently (which some hematology systems perform with proprietary agents), a true measure of this parameter alone would be insufficient.

Examining the light scattering of chicken blood is also insufficient to distinguish cell types. The correlation of both FSC and SSC with cell size and granularity is based on a spherical cell model with less correlation for non-spheroid cells (5). Loken *et al.* investigated scatter interpretation of asymmetrical cells in a flow system (47). The flattened, ellipsoid shape of avian erythrocytes results in light scattered over the entire range of intensity in both FSC and SSC detectors depending on the orientation of the cell to the illumination beam and detector. Forward scatter measurements generally fall into a bimodal distribution, with a continuum of cells perceived as of intermediate size. Side scatter, too, is dependent on orientation; the majority of cells fall in a peak of low SSC with the remaining cells extending into the uppermost channels. These distributions are a consequence of the amount of light reflected and bypassing these discoid cells in the interrogation point. This results in a spectrum of "size" and "granularity" that encompasses the entire range of channels,

effectively omitting any recognition of leukocyte populations based on these two parameters.

Cytochemistry

Several instruments differentiate leukocytes using cytochemical stains following erythrolysis. Peroxidase activity is revealed by adding a chromogen and hydrogen peroxide, then quantified using bright field detectors for absorbance (75,82,85-89,93). Hematology units have also used nonspecific esterase and Alcian blue reactivity to identify monocytes and basophils, respectively (19,28).

Various reports have attempted to elucidate the cytochemical reactions found in avian blood cells. These descriptions were often contradictory, and also demonstrated species differences. In a study by Atwal and McFarland (109), leukocytes of the Japanese quail (*Coturnix coturnix japonica*) were found to be devoid of cytochrome oxidase, peroxidase, alkaline phosphatase, acid phosphatase, acid ribonuclease, acid deoxyribonuclease, and succinic dehydrogenase activity. Erythrocytes, however, contained peroxidase, alkaline phosphatase, succinic dehydrogenase, and cytochrome oxidase activity. Others have noted a lack of myeloperoxidase activity in avian heterophils (102,110). A more recent investigation by Maiti *et al.* described α naphthyl acetate esterase activity in chicken lymphocytes, although other leukocytes were not examined (111). These findings were quite different from a limited cytochemical trial performed by the author, in which chicken eosinophils were positive for peroxidase, thrombocytes and monocytes for acid phosphatase, with no cellular reaction of chloroacetate esterase, alkaline phosphatase,

naphthyl-butyrate esterase, or acetylcholinesterase activity. This trial was not performed to repudiate the results stated in the literature, however, it is apparent that enzyme studies in chicken blood cells did not present supporting evidence that cytochemical methods would be of value for leukocyte enumeration. Additional descriptions of these activities may, however, identify methods of specifically identifying either erythrocytes or leukocytes.

Ultraviolet illumination

Many hematologic studies have affiliated alternative illumination wavelengths. The most commonly cited is the use of ultraviolet radiation (UV) and its effects on autofluorescence and light scattering. Erythrocytes have been discerned from leukocytes solely on their ability to absorb light in this spectrum (53). Leukocytes, as well, may have particular responses to UV illumination. Ultraviolet light has been employed to identify neutrophils based on the autofluorescence and scatter when examined with these wavelengths (107). Multiple wavelengths have been used simultaneously in units incorporating arc lamps as light sources (55).

An early instrument built by Curbelo *et al.* differentiated leukocytes and erythrocytes using five separate interrogation points for light scattered at various angles, fluorescence, and absorption (53). Erythrocytes were distinguished by the absorption of illumination at 420 nm by hemoglobin, which has a peak absorption at approximately 416 nm (72). Although a laser-driven unit created later gave poorer results (54), this parameter provides the most obvious form of specifically identifying erythrocytes and their precursors. Additionally, porphyrin precursors of the heme

molecule autofluoresce when excited by these wavelengths, resulting in emission of 575-600 nm (59). These type of analyses should function well regardless of the presence of an erythroid nucleus.

Leukocytes have also been identified by their autofluorescence when illuminated with certain wavelengths. Neutrophils have been enriched by examining their ability to autofluoresce when illuminated with UV light (107). This endogenous autofluorescence in the 460 nm region when excited between 350-360 nm examines cells for NAD(P)H (pyridine) fluorescence (56,58,112), which has been used as a measure of the oxidation-reduction state of cells (58). Additional components of autofluorescence are flavin-containing coenzymes, which emit blue 530 nm when excited by the 488 argon ion laser (56,57,113). Neutrophils have been studied by inducing a respiratory burst via illumination at 350 nm and detection at greater than 425 nm (114). Eosinophils are also reportedly "modestly" autofluorescent, comparable to approximately 50,000 molecules of FITC per cell (115). When illuminated with wavelengths in the 370-450 range, granule extracts demonstrate an emission maximum of 520 nm; this was used to purify eosinophils from a granulocyte-enriched suspension. This "eosinophil fluorescence factor" was not elucidated; however, major basic protein or peroxidase were not thought to be involved.

The use of these alternative wavelengths is dependent upon the instrumentation available. Many of the units currently manufactured for basic

research do not have the capability of measuring absorption or utilizing UV wavelengths for cellular illumination or detection.

Fluorescent dyes

Several fluorescent chemicals have been employed to preferentially stain mammalian leukocytes in automated instruments. For example, Shapiro *et al.* demonstrated a technique of distinguishing human leukocytes based on staining with ethidium bromide, brilliant sulfaflavine, a stilbene disulfonic acid derivative called "LN" ("long name") (54,72). The staining methodology, however, was sufficiently arduous to preclude its use in commercial hematology instruments.

Adams and Kamentsky recognized that leukocytes could be differentiated based on a one-step staining procedure with the metachromatic dye acridine orange (AO) (73). This fluorochrome has the ability to differentiate between single stranded RNA and double stranded DNA (116,117). In addition to it nucleic acid affinities, AO has also been demonstrated to stain leukocyte lysosomes, resulting in a red fluorescence (630-700 nm), that has been used for flow cytometric classification of mammalian leukocytes as lymphocytes, monocytes, and granulocytes (118-120). Although the use of AO in FCM requires rigid adherence to staining technique, this dye would likely be beneficial in examining avian blood cells. However, because of its affinity for plastic surfaces and potential contamination of subsequent analyses (117), AO is often not allowed in multi-use flow cytometers.

Two of the methods described in these works utilized fluorochromes to non-specifically label intracellular structures in avian blood cells. The third investigated immunological methods, which are commonly employed in FCM research.

Study Rationale

The primary objective of these investigations was to identify a means by which a standard commercial FCM could identify leukocytes in avian blood and provide leukocyte concentrations. White leghorn chickens, readily available as blood donors, were used as the model species. Various promising methods of differentiating chicken blood cells by induced fluorescence were identified and selected for further investigation. These included: 1) non-specific cytoplasmic protein staining by fluorescein isothiocyanate, 2) nucleic acid staining with thiazole orange, and 3) fluorescein-conjugated monoclonal antibody labeling of cytoskeletal proteins.

Fluorescein isothiocyanate

Although usually employed as a immunochemical label, fluorescein isothiocyanate (FITC) has also been used to stain nuclear and cytoplasmic protein (52,121-124). In high concentrations, leukocytes, with relatively higher levels of cytoplasmic protein, should absorb more FITC than erythrocytes, and therefore fluoresce with greater intensity. This can be evaluated by visual inspection using fluorescent microscopy to determine optimal staining concentrations (7).

Thiazole orange

Thiazole orange (TO) is a recently described nucleic acid stain that has been suggested for reticulocyte enumeration (125). This stain has many distinct advantages

over other nucleic acid dyes, such as superior fluorescence and membrane permeability. The nucleus of mature avian erythrocytes contains highly compacted chromatin (126), and would be expected to stain with less fluorescence intensity than other blood cells (46). Although mature chicken erythrocytes may contain pre-mRNA, the amounts are negligible when compared to actively transcribing cells such as leukocytes (127). Therefore, RNA in leukocytes was expected to contribute to an increased staining ability.

Cytoskeletal proteins

Immunological methods investigated the presence of three major cytoskeletal proteins found in mature avian erythrocytes: spectrin, vimentin, and tubulin (128).

Spectrin

Marchesi and Steers first termed the protein "spectrin," because of its discovery in erythrocyte ghosts, using antibodies which reacted with erythrocytes only (129). Spectrin has since been described as the most prevalent protein found in the membrane skeleton of erythrocytes, constituting up to 20% of the erythrocyte membrane-associated protein (128,130). In fact, most of the information known about the association of spectrin and the membrane skeleton arose from avian erythrocyte studies (131). This protein is made up of two subunits, α and β (132), which form a hexagonal lattice giving conformational stability to the plasma membrane and serving as a link between the membrane and other cytoplasmic proteins (128,130,131,133,134).

Antigenic similarities and differences between spectrin in various tissues have been identified in mammals and birds (133,135). The chicken α-spectrin subunit is strongly conserved among tissues (131), and has been found not only in host erythrocytes, but also neurons, epithelial cells, cortical cells of the lens, striated myocytes, endothelial cells, and connective tissue cells (136,137). Reactivity to this subunit is absent in chicken smooth muscle cells, embryonic fibroblasts, and spermatocytes, and most mammalian cells (137). The β-subunit is more tissue-specific, which led early immunological studies to conclude that the spectrin was not a ubiquitous protein (138). The three isoforms best characterized in chickens originate in erythrocytes, brain, or intestinal brush border cells (131,136). Cross-reactivity with avian tissues has been demonstrated with polyclonal antibodies raised against mammalian spectrin as well (133).

Vimentin

Intermediate filaments in the erythroid cytoskeleton are composed of vimentin subunits (128,139). These fibers suspend the nucleus in nucleated erythrocytes by attachments to the membrane skeleton and the nuclear membrane (140,141). These proteins contribute to the architecture maintaining the biconvex shape of the mature erythrocyte, and may also be involved in the nuclear retention in non-mammalian erythrocytes (128,139,142). Considerable homology has been reported between avian erythrocyte and muscle vimentin (143).

Tubulin

The conformation of microtubules in the avian erythrocyte has been the focus of a number of investigations. Microtubules play major roles in cell function and morphology (144). Their subunits, tubulins, are a homogeneous group of proteins that are evolutionarily conserved (145). Tubulins comprise approximately 0.6% of the protein in mature chicken erythrocytes. Most are found in a structure known as the marginal band (MB), which is composed primarily of tubulin (>95%), and accounts for roughly 0.45% of the erythrocyte protein content (146). The MB consists of several microtubules in cross-section. This represents either a few, or a single long, tubule(s) encompassing the equatorial rim approximately 50 nm beneath the plasma membrane (146). It has been identified in nucleated erythrocytes from birds, reptiles, amphibians, and fish (147), and is also found in thrombocytes (148-150). Immunologic homology between the thrombocyte and erythrocyte marginal band tubulin suggests that these two cell lineages are developmentally related (101,151). The MB is found in mammalian erythrocyte precursors and platelets (128,152-155); mature cells in species that contain elongated erythrocytes, such as camels, also contain this structure (156).

The MB originates as a disorganized collection of microtubules around the erythroblast nucleus, which progresses to a wreath-like structure in polychromatophilic erythroblasts, and finally to a ribbon-like band around the equator of the mature erythrocyte (151). Unlike other microtubule bundles, there is apparently

no involvement of a microtubule-organizing center involved in support of the mature MB (145).

The major role of the MB is presumably maintenance of the erythrocyte oval shape (128,156,157), especially in the developing cell (147). The tension produced by the membrane skeleton alters the preferred circular shape of the MB into an oblong conformation (158).

Microtubules are dimers composed of α- and β-subunits (159). The tubulin β-subunits of the MB in chicken erythrocytes differ immunologically from other microtubules in cells such as neurons (151). A low degree of homology has also been observed between the β-tubulin in mammalian MB and cells from other tissues (152). This specificity occurs gradually over the development of cells as the tubulins are incorporated into the MB (151). Tubulins in other cells do not react with antibodies to this specific subunit. This lack of homology may be related to the function of these microtubules in different cell types (160).

Immunofluorescent identification

Although non-erythroid spectrin has been identified in mammalian monocytes (133), the structural protein differences in avian leukocytes and erythrocytes are not well documented. Descriptions of the ultrastructure of chicken leukocytes by electron microscopy note the absence of the MB in leukocytes (148-150), although microtubules associated with centrioles have been observed (150). However, there are no studies that address the distinction of cellular structural proteins in avian erythrocytes, thrombocytes, and leukocytes. These investigations sought a

quantitative difference in cytoskeletal staining between these major populations of blood cells using a commercially available polyclonal antibody directed against chicken spectrin (Sigma Immunochemicals, Sigma Chemical Company, St. Louis, MO), and monoclonal antibodies against vimentin (BioGenex Laboratories, San Ramon, CA) and β-tubulin (Sigma Immunochemicals, Sigma Chemical Company, St. Louis, MO).

There are two methods of performing immunofluorescence studies: direct and indirect. Direct techniques utilize a single, fluorochrome-labeled antibody; indirect methods use a primary antibody directed against the antigen of interest with a labeled secondary immunoglobulin directed at the primary antibody (5,161). Direct methods are more rapid due to the omission of secondary antibody step. However, inconsistent conjugation of fluorochrome to the primary antibody can result in increased staining variability (162), and more cost associated with the amount of fluorochrome necessary (9). Indirect methods are more sensitive, between 4- and 10-fold, and can be less expensive due to the availability of commercially-produced secondary antibody (9,162). Many antibodies are limited in their availability; loss of antibody concentration during conjugation is prevented using a common, relatively easily obtained, secondary antibody (163).

Expected Limitations

With the presumption that leukocytes and thrombocytes will be preferentially stained with these various fluorochromes or markers, a major limitation remains. Most hematological instruments remove the erythrocytes from the leukocyte

"channel," which increases the ability of the instrument to discern the remaining cells. The following studies attempted to distinguish leukocytes and thrombocytes from erythrocytes in whole blood. According to Lucas and Jamroz, the average erythrocyte count in adult chickens is approximately 3.0 x $10^6/\mu L$; the average leukocyte count 26 x $10^3/\mu L$; and the average thrombocyte count 44 x $10^3/\mu L$ (164). Leukocytes therefore compose only 0.87% of the total cell count, or 2.3% including thrombocytes, which can be considered a rare cell analysis. Although several authors have cited the theoretical ability of FCM to discriminate rare populations, from 5% (43) to 0.001% (5), the precision of recognizing these cells remains uncertain. Interference by debris and doublets (conjoined cells) that may be perceived as larger or more brightly stained cells will play a much greater role when examining these relatively small populations (43).

Another recognized concern is the restricted ability of the FCM employed in these studies (FACScan™, Becton-Dickinson, San Jose, CA) to perform quantitative analyses on rare cells. This instrument is designed to compare large populations and calculate relative amounts. Converting this data to an absolute concentration will necessitate the use of fairly crude methods such as analysis with a given flow rate or comparison to total cell counts obtained by hemacytometers.

Nonetheless, several manufacturers have indicated a desire to modify existing hematology instruments for non-mammalian blood. Various laboratories are also investigating the ability of currently available units for analyzing of avian specimens (77). This acknowledged exigency for automated hematology in birds, reptiles,

amphibians, and fish, predicates the demand for investigating methods adaptable to these species.

Chapter 2: Flow Cytometric Differentiation of Avian Erythrocytes and Leukocytes with Fluorescein Isothiocyanate

Introduction

The complete blood count (CBC) is a primary diagnostic test for evaluating diseased animals; a fundamental parameter of the CBC is the total leukocyte count or concentration. Currently, there are three manual methods available for determining the leukocyte count in avian and other non-mammalian species: indirect, semi-indirect, and direct. The indirect method estimates the number of leukocytes in a blood smear by comparison to the number of erythrocytes. Direct methods utilize special stains to derive an absolute leukocyte count by visualization in a hemacytometer. The semi-indirect method, which is currently the most popular (77), utilizes phloxine stain to identify the heterophils and eosinophils, thereby allowing their summation in a hemacytometer. The leukocyte concentration must then be calculated by a correlation to the percentage of these granulocytes in the differential cell count (96).

Disadvantages to these methods are numerous. The relatively small sample size generates the major source of error (68). Although acceptable precision can be achieved with a high number of phloxine-positive cells (101), this can be negated by the inaccuracy associated with the use of hemacytometers (105). In both the indirect and semi-indirect methods, proper slide preparation is vital. Minor anomalies in blood smears can create marked differences in the total leukocyte count (96,102).

Automation has resulted in vast improvements of precision and accuracy in mammalian hematology. Expenses may also be reduced, especially in technical training and labor (19,68,94). Unfortunately, non-mammalian blood cannot be evaluated using current instruments due to the nucleus found within the erythrocytes and thrombocytes (102).

Hematologic instrumentation is based on the technology of flow cytometry (FCM). These machines utilize a system of fluidics to reduce a suspension into a single stream of cells. Lasers or strong incandescent sources provide a light beam, which strikes individual cells as they pass a set point along the path of flow, called the interrogation point. Signals, such as light scatter and fluorescence, are then detected and analyzed (4). Properties such as size, density, and fluorescence of bound dyes have been used to classify subpopulations in a heterogenous sample (7). Because of the speed at which cells are analyzed, as well as the sensitivity of the detection systems, FCM has been utilized extensively in analytical cytometry, both in clinical hematology and basic science research (5). An additional feature of many instruments is the ability to physically collect different populations of cells after the interrogation has occurred in a process known as cell sorting. Sorters apply a charge to cell-containing droplets and divert those of interest from the main stream in an electric field. This allows purification of cell types based on any measurable parameter (11).

Fluorescein isothiocyanate (FITC) has long been recognized for its utility in fluorescence cytometry. Although frequently associated with labeling immunological markers, FITC also binds non-specifically to cellular proteins. This property has been

used to estimate cytoplasmic (52,121-123) and nuclear (124) protein content. The isothiocyanate moiety of FITC facilitates protein conjugation (165) with a covalent bond to the ε-amino group of lysine (166). Its excitation with 488 nm and fluorescence emission peak of 530 nm conforms well with the lasers and detectors in most FCM units (11,121).

The following investigations utilized commercial FCM instruments (FACScan™ and FACS 440™, Becton-Dickinson Immunocytometry Systems, San Jose, CA) to evaluate the use of this fluorochrome in avian blood. The primary objective was to determine if staining cytoplasmic and nuclear protein with FITC would sufficiently differentiate chicken erythrocytes and leukocytes, and thereby establish a means of calculating the total leukocyte count. Due to the variety of proteins contained in leukocyte cytoplasm, and the homogeneity in erythrocytes which contain essentially only hemoglobin, leukocytes were expected to preferentially bind more FITC. The binding of nuclear protein may also be greater in leukocytes. Access to the chromatin-associated proteins plays a role in fluorescence intensity of stained cells (167). Erythrocytes, which contain more densely clumped chromatin, therefore have less nuclear protein accessible. The majority of the erythrocytes could be ignored by creating a minimum FITC fluorescence threshold. A generated gate or region encompassing the leukocytes would then permit the leukocyte concentration calculation given the cell count per unit time and flow rate.

Hematology instruments generally lyse erythrocytes prior to examination of leukocytes. Applied to these studies, this procedure would serve to limit available cell

substrate to leukocytes, further increasing the likelihood of specific staining. However, commercially available agents rarely lyse all avian erythrocytes without also damaging leukocytes (168). Limited studies performed by the author using several erythrocyte lysing solutions produced similar findings, i.e. sufficient erythrolysis also resulted in considerable leukocyte lysis. In additional investigations, blood cells were subjected to various concentrations of hypotonic saline to determine the effect of cell swelling and lysis on FITC staining proclivity.

Materials and Methods

Blood collection

Adult white leghorn hens from the Louisiana State Agricultural Experimental Station, Central Station - Poultry Unit were used as blood donors. Approximately 5 mL of blood was drawn from the cutaneous ulnar vein into K_2EDTA-coated syringes with 25 gauge needles. The blood was then immediately transferred to K_3EDTA collection tubes (Vacutainer®, Becton Dickinson Vacutainer Systems, Rutherford, NJ).

Blood separation

Whole blood samples were created by diluting 300 µL whole blood in 200 µL 1.0 M phosphate-buffered saline (PBS) for a final concentration of approximately 1.3 x 10^6 cells/µL in 1.5 mL micro-centrifuge tubes (Dot Scientific, Inc., Burton, MI). Cell concentrations were performed using a commercial hematology instrument (Mascot™ XE, CDC Technologies, Inc., Oxford, CT) using the erythrocyte channel. The remaining blood was centrifuged (Beckman® Model YJ-6R, Beckman, Palo Alto, CA) at 200 *g* for 30 minutes. Erythrocyte-enriched samples were prepared by

suspending 100 μL of this pellet in 400 μL PBS for an approximate concentration of 1.3×10^6 cells/μL. The plasma, buffy coat, and upper erythrocyte pellet were removed and placed into 1.0 mL hematocrit tubes (Seditube™, Becton-Dickinson, Rutherford, NJ) and further centrifuged at 200 *g* for 30 minutes to create a more prominent buffy coat. The buffy coat and plasma were removed from the hematocrit tubes and resuspended in 500 μL PBS to generate the leukocyte-enriched samples.

Hypotonic lysis

For initial experiments, 50 μL whole blood was diluted in 5.0 mL of various different concentrations of NaCl (0.0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, and/or 0.9%) as used to perform osmotic erythrolysis (169,170). Additional samples were diluted at 1:10 (50 μL blood in 0.5 mL). These samples were incubated for 30 minutes at RT, and then centrifuged at 200 *g* for 10 minutes. A drop of new methylene blue stain was added to the resulting pellet and placed on a slide for microscopic examination.

A total granulocyte count was performed as described by Wiseman (103), on the whole blood, 0.2%, 0.3%, and 0.9% diluted blood from two birds to determine the effects of hypotonicity on the leukocyte count. This was accomplished using a phloxine dye kit used for human eosinophil counts (Unopette® Test 5877, Becton-Dickinson, Vacutainer Division, Rutherford, NJ), which stains avian heterophils and eosinophils. These cells were counted in a hemacytometer with Neubauer ruling.

For subsequent studies, 50 μL of each sample (whole blood, erythrocyte-, and leukocyte-enriched) were added to 0.5 mL of each concentrations of saline. These were incubated at RT for approximately 30 minutes.

Stain preparation and use

For fluorescent microscopy experiments, a stock solution of 1 mg FITC (fluorescein 5-isothiocyanate, Sigma Chemical Co., St. Louis, MO) per mL acetone was prepared and frozen at -10°C as described by Miller and Quarles (123). Working concentrations of 250, 25, and 2.5 μg/mL were made in phosphate-buffered saline (PBS). For FCM studies, staining solutions were prepared fresh daily from the original FITC powder. Samples were stained with 100 μL of the three concentrations of FITC and incubated for 30 minutes at 4° C. Excess stain was removed by centrifugation at 200 *g* for 10 minutes, and washing of pellets in PBS twice. Finally, the cells were resuspended in 500 μL PBS and further diluted for FCM analysis: 1:100 for erythrocyte-enriched and whole blood samples, and 1:20 for leukocyte-enriched samples. These were placed on glass slides; coverslips were mounted using an aqueous media (Immu-mount, Shandon, Pittsburgh, PA) for fluorescent microscopy, or in polystyrene tubes (Falcon® 2054, Becton-Dickinson Labware, Becton Dickinson and Company, Lincoln Park, NJ) for FCM analysis.

Fluorescent microscopy

Incident light fluorescent microscopy (Ortholux II™, Leitz Wetzlar, E. Leitz, Inc., Rockleigh, NJ) utilized a 200 watt high-pressure mercury vapor lamp as a light source (Model 050222, E. Leitz, Inc., Rockleigh, NJ). This light source was equipped

with a multi-wavelength fluorescence illuminator (Pleomopak, E. Leitz, Inc., Rockleigh, NJ). Barrier and exciter filters were inserted in the light path to exclude shorter blue wavelengths to maximize fluorescein emission detection.

Flow cytometry

Flow cytometric analysis of the separated blood samples was performed with a commercial instrument (FACScan™, Becton Dickinson Immunocytometry Systems, Becton, Dickinson, and Co., San Jose, CA). This unit used a 15 mW, air-cooled, 488 nm argon ion laser and sterile physiologic saline as sheath fluid. Detection was supplied by photomultiplier tubes as: FL-1 (green fluorescence), FL-2 (orange fluorescence), FL- 3 (red fluorescence), SSC (side-scatter), and FSC (forward scatter). Fluorescence detectors were amplified logarithmically and incorporated a 530/30 nm and 585/42 nm band pass filter for green and orange, and a 670 nm long pass filter for red fluorescence. Data was acquired in list mode to a computer interface (Hewlett-Packard HP 9000 series, model 340, San Diego, CA) and analyzed using supplied software (Lysys™ II, Becton Dickinson Immunocytometry Systems, Becton Dickinson and Company, San Jose, CA).

The cell sorting instrument (FACS 440™, Becton Dickinson Immunocytometry Systems, Becton, Dickinson, and Co., San Jose, CA) was equipped with an argon-ion laser (Coherent, Palo Alto, CA) operated at 200 mW with excitation at 488 nm. Detection methods were similar to the FACScan™, using band pass filters (530/30 and 585/42) for green and orange fluorescence, respectively. Physiological saline sheath fluid was maintained at 10 psi, with a drive frequency of 23-25 kHz,

nozzle orifice of 70 μm in diameter, and a sorting rate of 1000-1300 cells per second. Data was acquired from two chickens and collected onto a workstation (DEC MicroVAX II, Digital Corporation, Maynard, MA) and analyzed with the supplied software (Consort 40, Becton Dickinson, San Jose, CA).

Sorting was based on a green fluorescence intensity threshold, with cells separated corresponding to events on the right and left of the threshold corresponding to higher and lower fluorescence. This threshold was placed at various intervals along the sample distribution (Figure 2.1). Cells were sorted into siliconized glass tubes (25 x 7 mm) which were coated with approximately 0.1 mL of filter-sterilized chicken plasma. Slides were prepared from these samples using a cytocentrifuge (Cytospin®, Shandon Southern Instruments, Inc., Sewickley, PA) and stained using a modified Wright's stain in an automatic slide stainer (Hema-tek® Model 4480, Ames Company, Division of Miles Laboratories, Elkhart, IN).

Results

Fluorescent microscopy

Examination of slides of FITC-stained blood cells from two chickens by fluorescent microscopy revealed that identifiable leukocytes fluoresced greater than erythrocytes (Figure 2.2). This difference in fluorescence was greatest at the 25 μg/mL FITC concentration. In the erythrocyte-enriched samples, many unidentifiable cells also stained brightly with FITC, with approximately 20 - 30 per low power (100x) field. Wright's stains of these preparations identified many granulocytes, at approximately the same concentration as observed by fluorescence. Smears of the

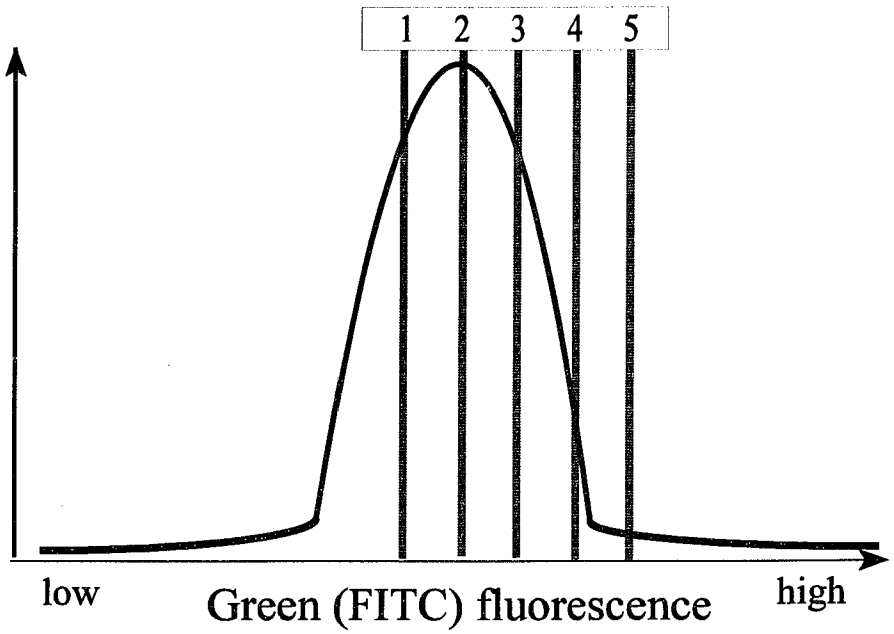

Figure 2.1. Schematic frequency histogram of green fluorescence illustrating placement of cell sorting thresholds. Whole blood samples were sorted and collected according to their relative fluorescence to the right or left of the threshold for each of five successive sorts.

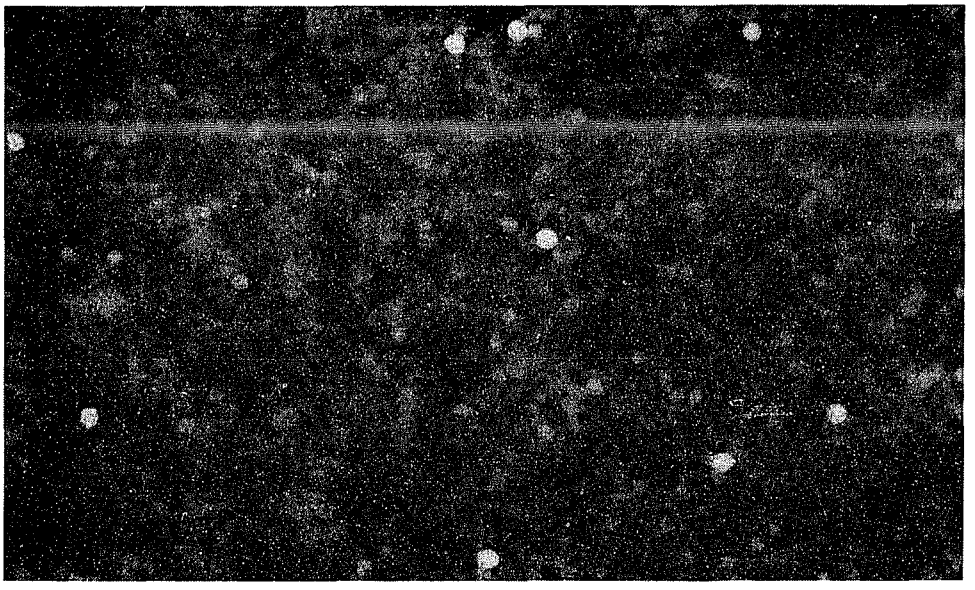

Figure 2.2. Fluorescent micrograph of chicken whole blood stained with 25 μg/mL FITC. Note the presence of low numbers of cells with higher fluorescence intensity.

leukocyte- and erythrocyte-enriched samples revealed fairly well-preserved cells, i.e. representatives of all leukocyte series were identifiable, as were a number of "smudge" cells.

Flow cytometric analysis

Separated blood samples

Flow cytometric analysis was performed on separated blood samples from two different chickens. Graphical displays were essentially identical in both samples. Based on the frequency histogram of FITC fluorescence as detected by FL-1, the leukocyte-enriched samples demonstrated a peak shifted significantly to the right of the erythrocyte-enriched peak (Figure 2.3). This difference was greatest using concentrated stains (250 μg/mL), but was present in the lower concentrations as well. Mixed samples (one drop erythrocyte-enriched + one drop leukocyte-enriched preparations) contained a shoulder of increased fluorescence corresponding to that seen in the leukocyte-enriched peak (Figure 2.4).

Erythrocyte-enriched cells exhibited greater fluorescence than leukocyte-enriched samples in the red spectra as detected by FL-3. This was found in unstained samples, and those stained at the 2.5 and 25 μg/mL FITC. At 250 μg/mL, the populations became confluent (Figure 2.5).

The fluorescence events were also examined using multiple parameters simultaneously. Using the inverse relationship of FL-1 and FL-2 fluorescence in erythrocyte- and leukocyte-enriched samples improved discrimination of two populations identified by the regions X and Z (Figure 2.6). This segregation was

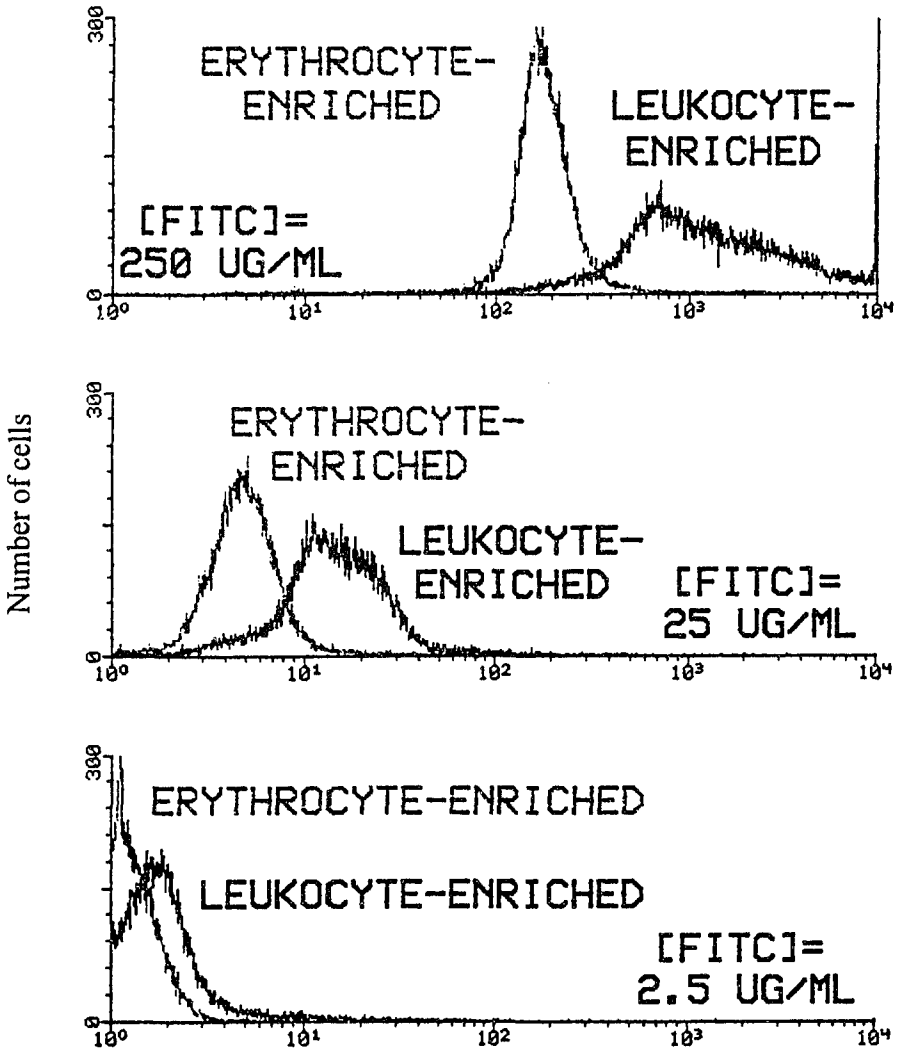

Figure 2.3. FL-1 fluorescence frequency histograms of erythrocyte- and leukocyte-enriched chicken blood samples stained with FITC at 2.5, 25, and 250 µg/mL.

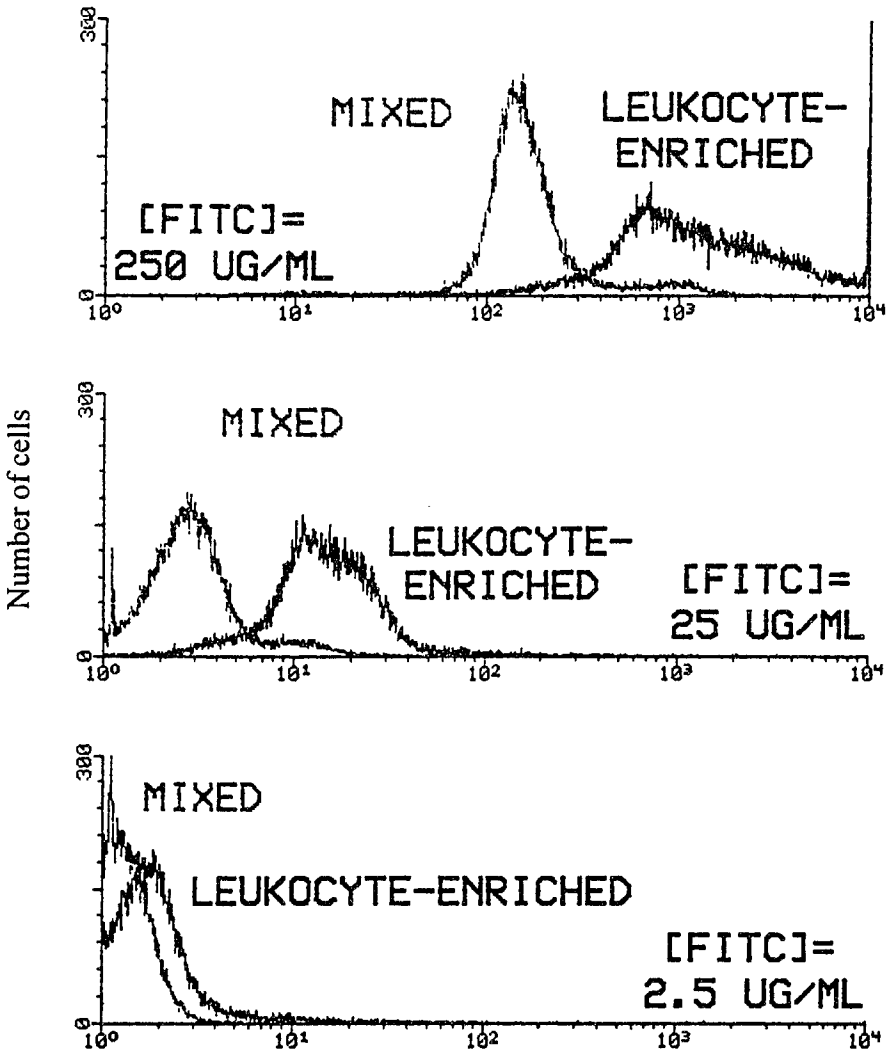

Figure 2.4. FL-1 fluorescence frequency histograms of mixed and leukocyte-enriched chicken blood samples stained with FITC at 2.5, 25, and 250 μg/mL. Note the shoulder of higher intensity events in mixed samples corresponding to the primary peak in leukocyte-enriched sample distribution.

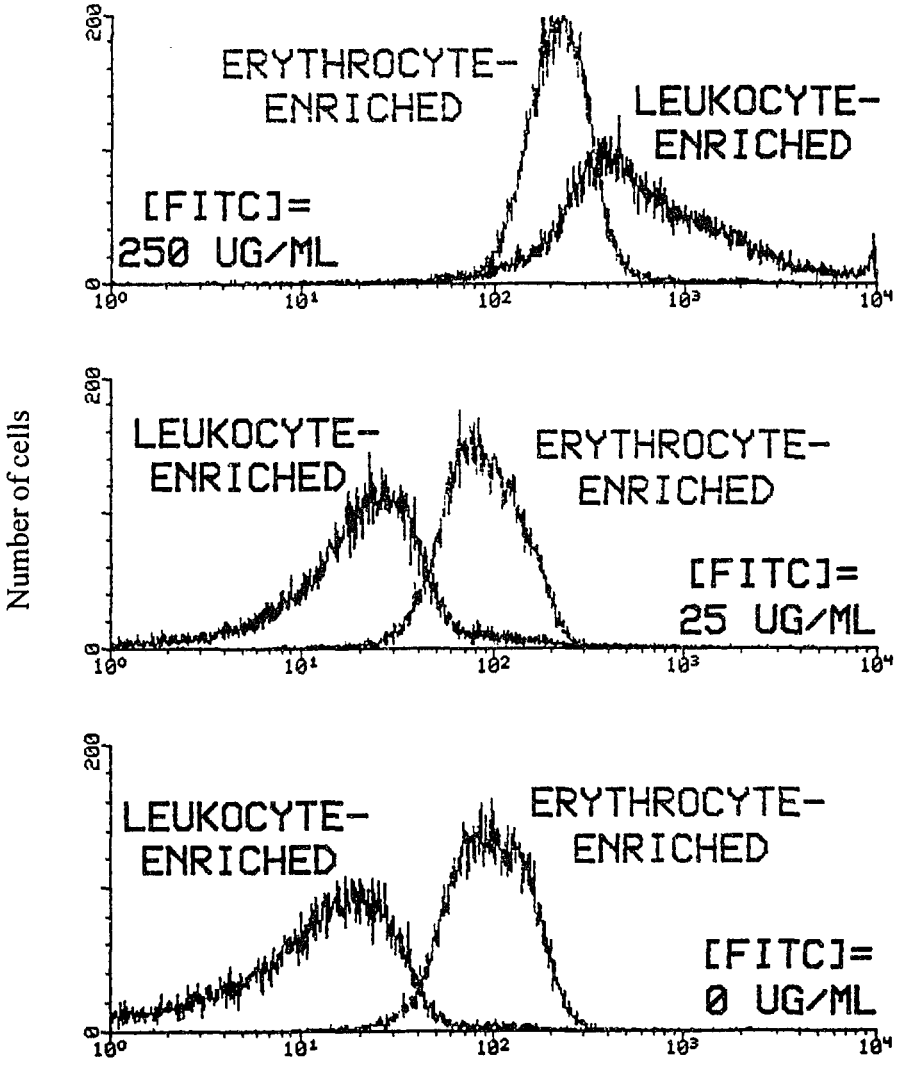

Figure 2.5. FL-3 fluorescence frequency histograms of erythrocyte- and leukocyte-enriched chicken blood samples stained with FITC at 0, 25, and 250 μg/mL.

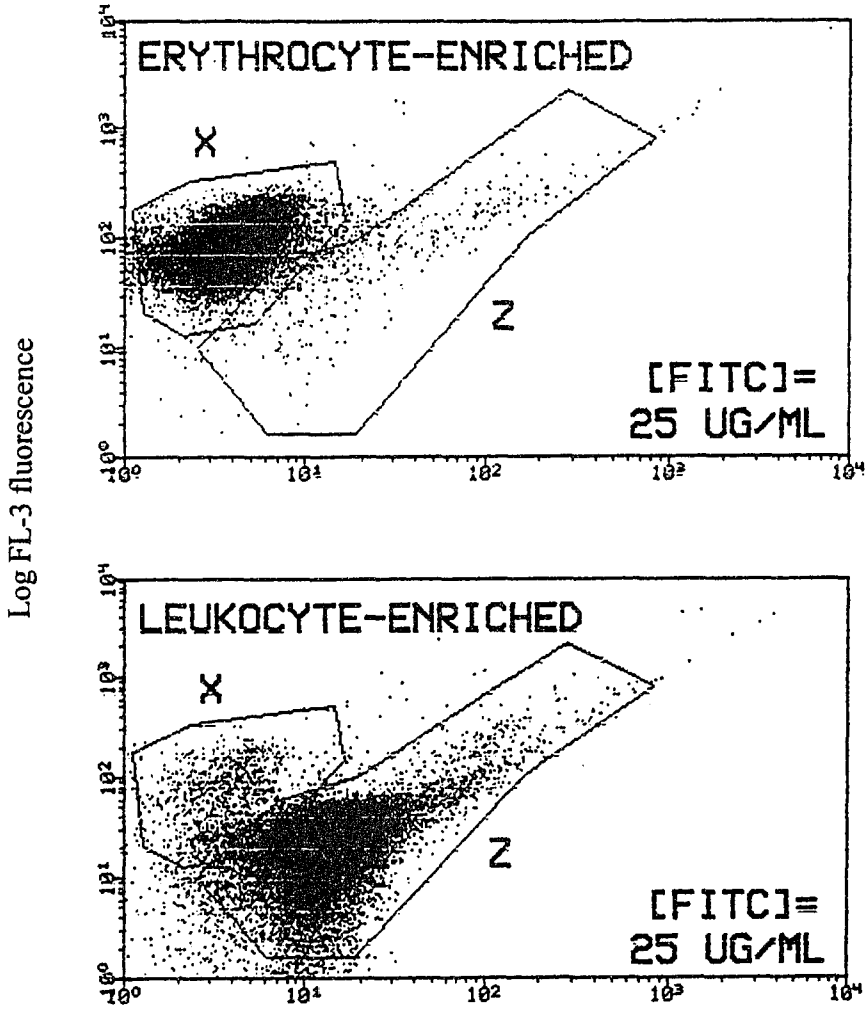

Figure 2.6. Two parameter analysis (FL-1 vs. FL-3) of erythrocyte- and leukocyte-enriched chicken blood samples stained with 25 μg/mL FITC. Regions X and Z were drawn to encompass the majority of events in the erythrocyte- and leukocyte-enriched samples, respectively.

greatest in cells stained with 25 μg/mL FITC. At 2.5 μg/mL, FL-1 intensities were confluent, at 250 μg/mL, FL-3 intensities overlapped (Figures 2.3 and 2.5). Region X was drawn to encompass the majority of events in the erythrocyte-enriched samples; region Z circumscribed the primary populations in the leukocyte-enriched samples.

Hypotonic blood cell lysis

Grossly, hemolysis was present in the supernate in blood samples diluted in 0.0-0.4% saline. In the 0.0% and 0.1% samples, white clumps remained after the initial washing step. These aggregates prevented FCM analysis of these samples by causing temporary clogging of the fluidics system. Microscopic evaluation of hypotonically lysed whole blood samples revealed clumps of nuclei with no discernable intact cells in the 0.0% and 0.1% samples. The 0.2% samples consisted mostly of bare nuclei, although some leukocytes were seen. In the 0.3% samples, few intact erythrocytes, but representatives of all leukocyte series, were identified. In 0.4% samples, the majority of the erythrocytes were also undamaged. In 0.5-0.9% NaCl, all cells were intact and no hemoglobin was noted in the supernate.

Total leukocyte counts were not possible using 1:100 dilution of blood because of the scarcity of granulocytes; therefore, a 1:10 dilution was examined. The number of phloxine-stained cells in the hemacytometer were similar (corrected for dilution) in all samples: 33 and 38 for whole blood; 33 and 25 in 0.9%; 32 and 36 in 0.3%; and 23 and 25 in 0.2%. The similarity of these numbers was regarded as evidence that hypotonic lysis did not significantly alter the leukocyte numbers.

Because of the degree of erythrocyte lysis and minimal effects on leukocytes, 0.3% NaCl was selected as the diluent for FCM analysis of enriched samples using FL-1 histograms and FL-1 vs. FL-3 dot-plots. Flow cytometric analysis was performed on hypotonically lysed blood cells from three chickens, resulting in essentially identical results as evident from graphical analysis. Both erythrocyte- and leukocyte-enriched samples increased in fluorescence intensity when exposed to the hypotonic saline; however, the resolution between leukocyte- and erythrocyte-enriched samples remained unimproved (Figure 2.7). In a similar study, several whole blood, erythrocyte- and leukocyte-enriched samples displayed a bimodal distribution (Figure 2.8). These were compared to similar samples diluted in 0.9% NaCl and stained with FITC. A significant peak in the whole blood and erythrocyte-enriched sample distributions corresponded to the higher FITC staining intensity seen in the leukocyte-enriched samples. A notable peak in the leukocyte-enriched samples also remains in the low intensity region similar to that seen in the whole blood and erythrocyte-enriched samples.

To investigate this further, samples suspended in several concentrations of saline were analyzed to determine the effects of varied tonicity. These were compared using FL-1 vs. FL-3 dot plots (Figures 2.9, 2.10, and 2.11). Regions X and Z were again created to encompass the majority of the cells seen in the erythrocyte- and leukocyte-enriched samples diluted in 0.9% NaCl. The number of events occurring in region Z increased in relative size in decreasing concentrations of diluent.

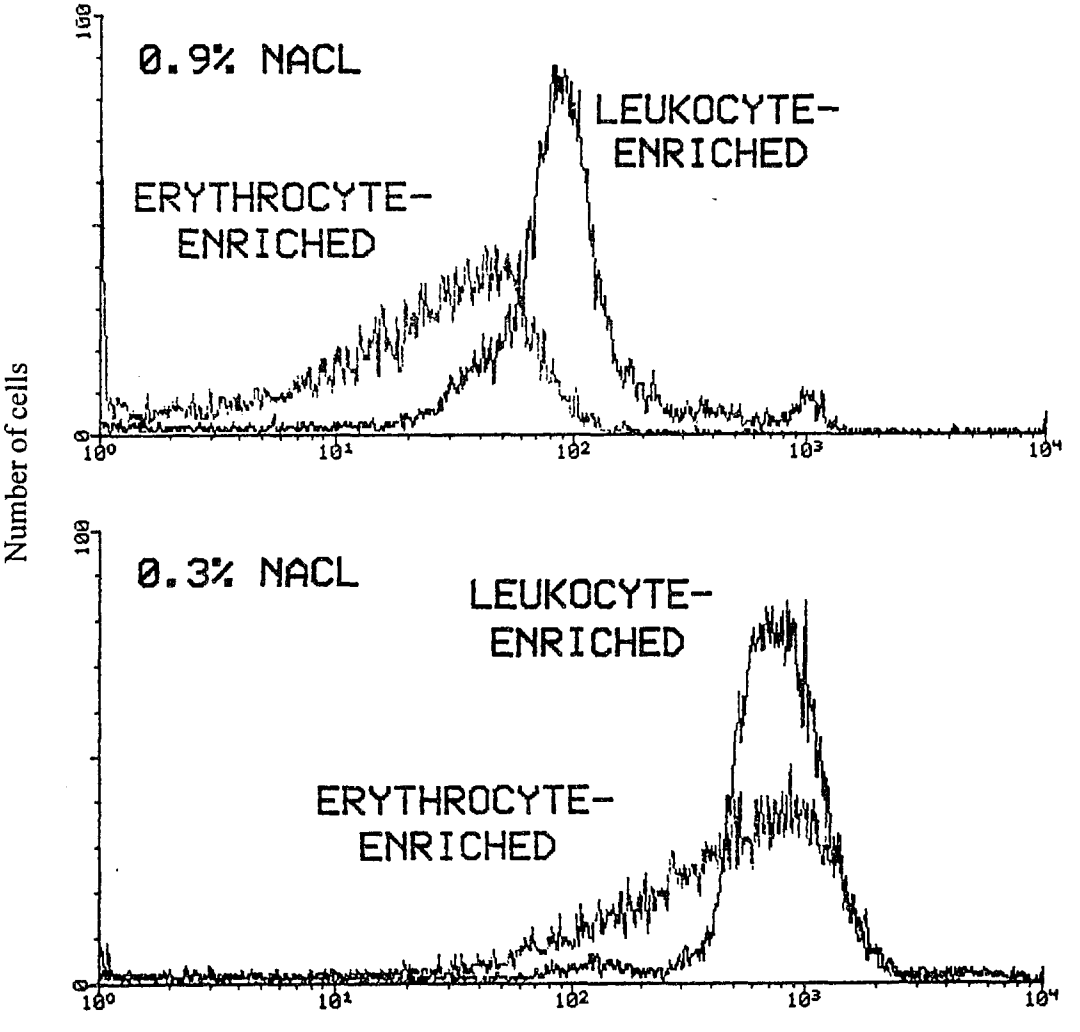

Figure 2.7. FL-1 fluorescence frequency histograms of erythrocyte- and leukocyte-enriched chicken blood samples stained with 25 μg/mL FITC after dilution in either isotonic (0.9%) or hypotonic (0.3%) saline.

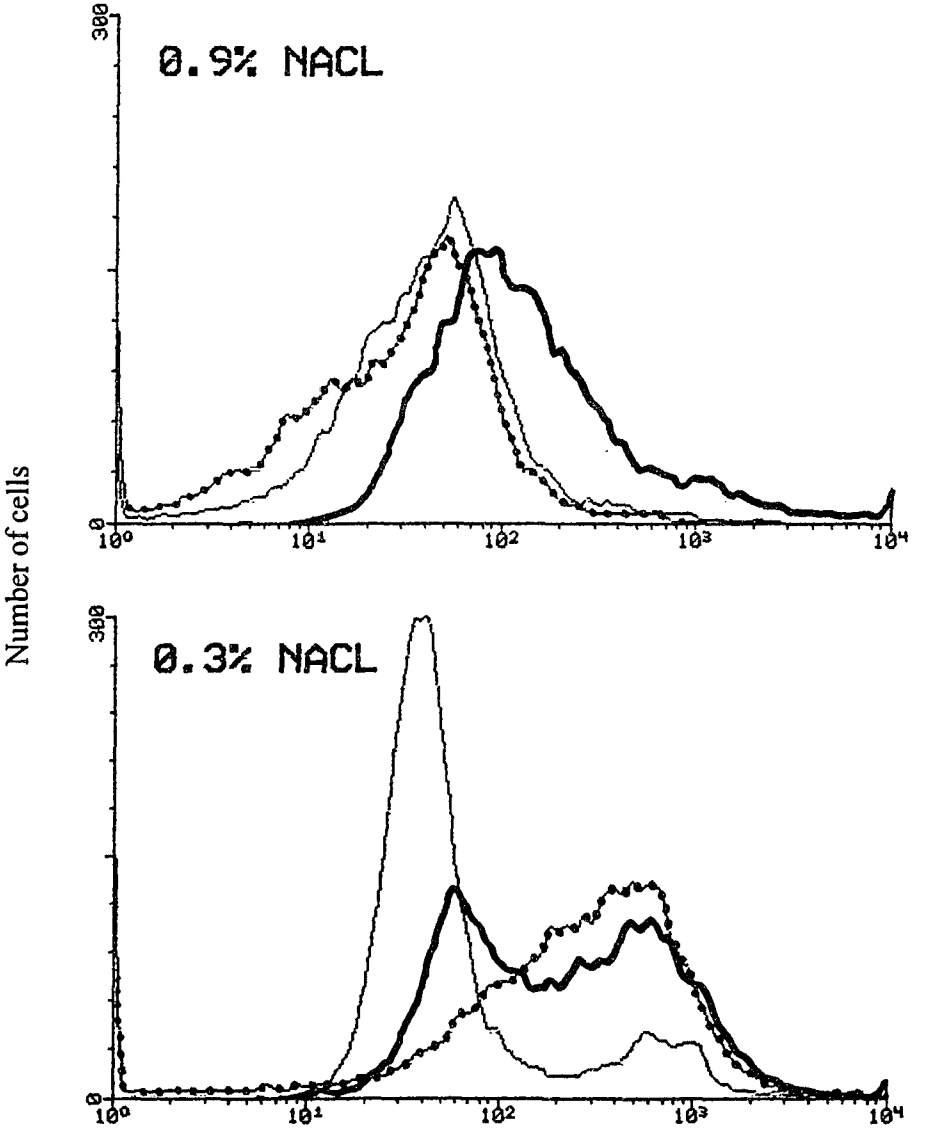

Figure 2.8. FL-1 fluorescence frequency histograms of whole, erythrocyte-, and leukocyte-enriched chicken blood stained with 25 μg/mL FITC after dilution in either isotonic (0.9%) or hypotonic (0.3%) saline. Note two significant peaks in whole and leukocyte-enriched samples in 0.3% NaCl with the lower peak corresponding to that seen in 0.9%.
Legend = whole (--------), erythrocyte-enriched (-•-•-•-), leukocyte-enriched (▬▬▬)

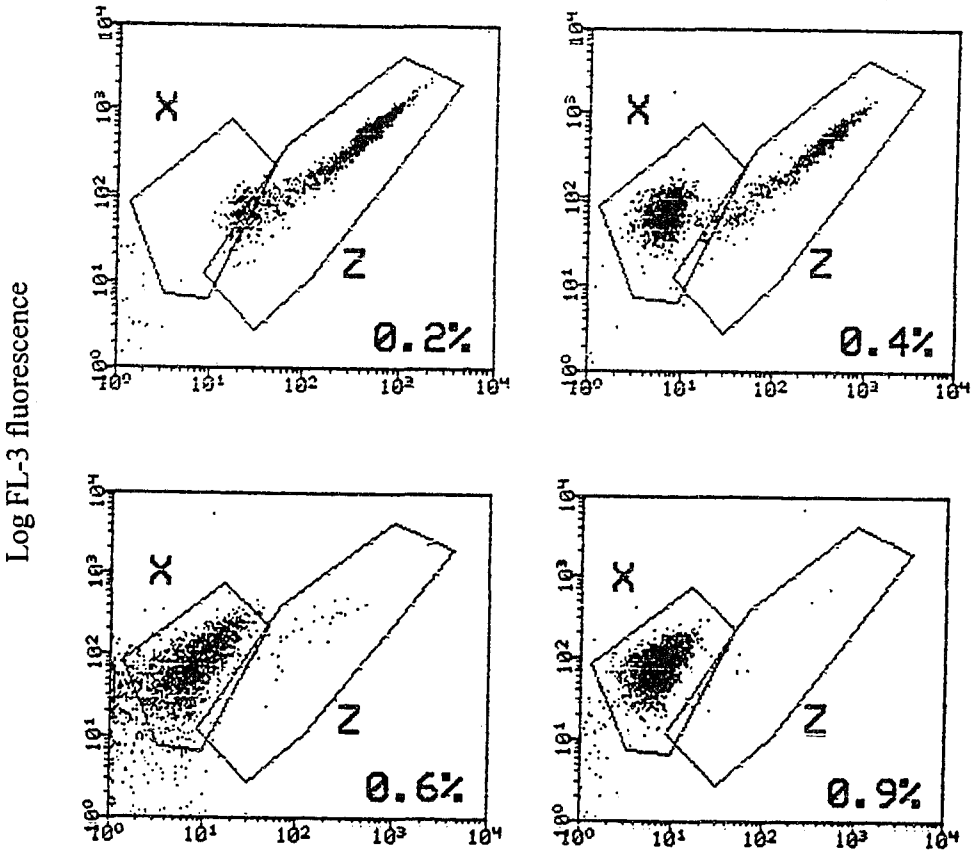

Figure 2.9. Two parameter analysis (FL-1 vs. FL-3) of erythrocyte-enriched chicken blood samples stained with 25 μg/mL FITC after dilution in either 0.2%,0.4%, 0.6%, or 0.9% NaCl solution. Regions X and Z were drawn to encompass the majority of events in erythrocyte- and leukocyte-enriched samples in the 0.9% NaCl, respectively. Note the relative increase of events in region Z in diluents of decreased tonicity.

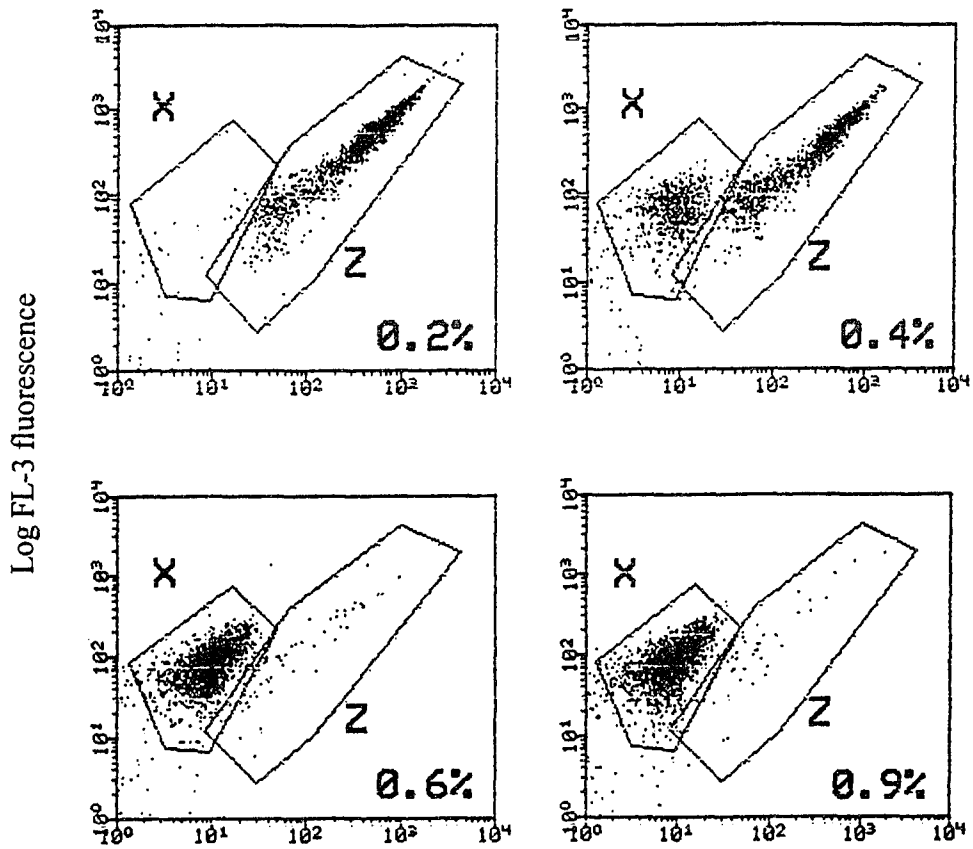

Figure 2.10. Two parameter analysis (FL-1 vs. FL-3) of chicken whole blood samples stained with 25 μg/mL FITC after dilution in either 0.2%, 0.4%, 0.6%, or 0.9% NaCl solution. Regions X and Z were drawn to encompass the majority of events in the erythrocyte- and leukocyte-enriched samples in 0.9% NaCl, respectively. Note the relative increase of events in region Z in diluents of decreased tonicity.

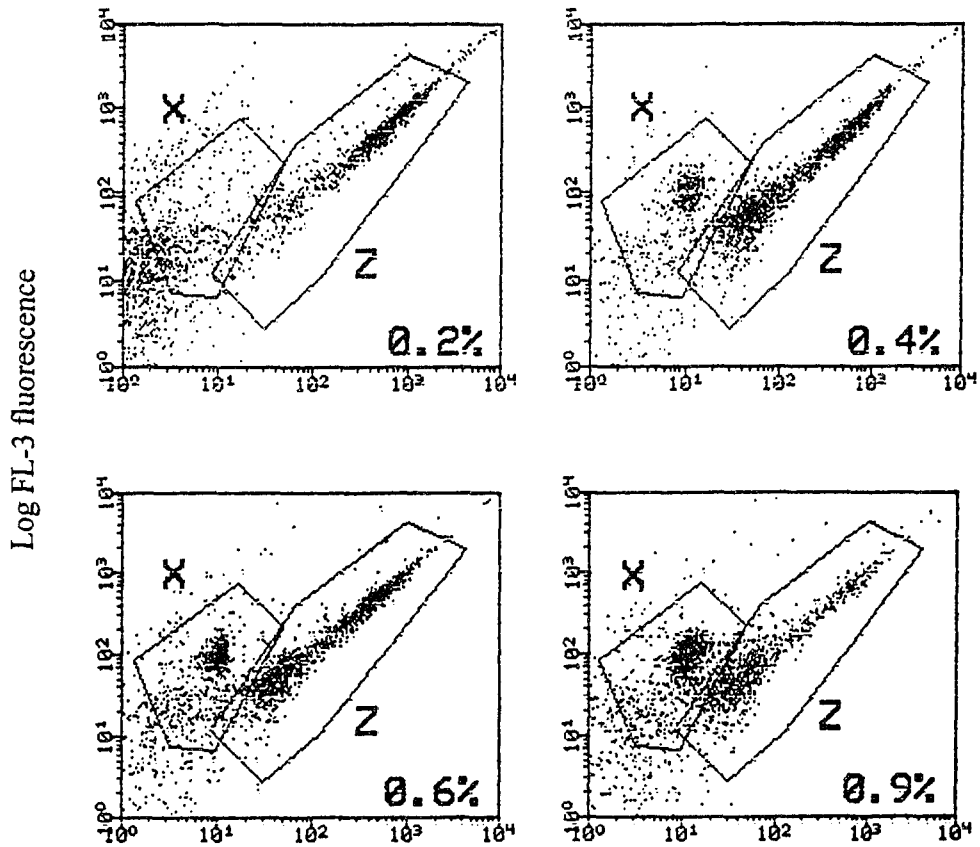

Figure 2.11. Two parameter analysis (FL-1 vs. FL-3) of leukocyte-enriched chicken blood samples stained with 25 µg/mL FITC after dilution in either 0.2%, 0.4%, 0.6%, or 0.9% NaCl solution. Regions X and Z were drawn to encompass the majority of events in the erythrocyte- and leukocyte-enriched samples in 0.9% NaCl, respectively. Note the relative increase of events in region Z in diluents of decreased tonicity.

Cell sorting

Microscopic examination of the cells in sorts number 4 and 5 (Figure 2.1), the majority of the cells falling to the rights of the threshold, i.e. high green fluorescence, were leukocytes. However, leukocytes and erythrocytes were evident in the right and left sides of all thresholds. Although relative concentrations were not calculated, the number of leukocytes collected in the left decreased as the threshold was moved to the left (lower green intensity).

FITC lability and decay

It was observed during fluorescent microscopic investigations that the FITC stain solutions were labile. Staining samples with solutions made on the previous day yielded significantly poorer staining at the two lower concentrations (2.5 and 25 μg/mL).

A FCM study illustrated FITC fluorescence decay. Samples from two chickens were stained at the same time; however, samples collected at the later time of 16:44 displayed approximately half of the peak fluorescence when compared to those collected 1⅓ hours previously (Figure 2.12).

Discussion

Centrifugation did not yield completely separated populations of erythrocytes and leukocytes. Examination of purified, rather than enriched, samples may have permitted recognition of both low fluorescence intensity leukocytes and high fluorescence intensity erythrocytes discovered in sorted preparations earlier in the study. Additionally, by removing erythrocytes from a particular layer in centrifuged

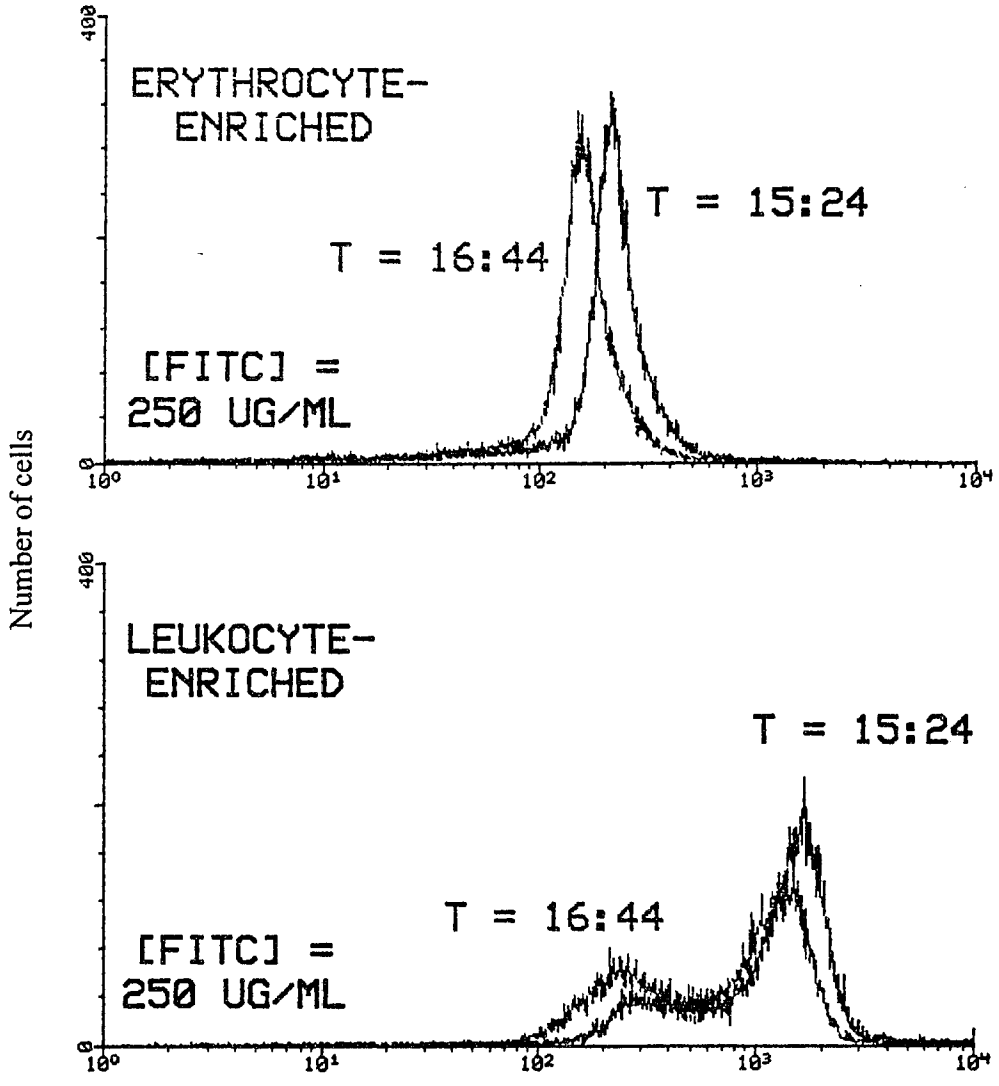

Figure 2.12. FL-1 fluorescence frequency histogram of erythrocyte- and leukocyte-enriched chicken blood samples stained concurrently with 250 µg/mL FITC analyzed at time 15:24 and 16:44.

blood, erythrocytes with a specific density were likely selected. These cells may not have fluorescent properties representative of the total erythrocyte population.

Microscopic observation of increased FITC fluorescence by leukocytes correlated with the distribution of high FL-1 intensity in the leukocyte-enriched samples (Figure 2.3). However, the amount of overlap with the distribution of lower FL-1 intensity events predominating in the erythrocyte-enriched samples prevents the assessment of leukocyte concentration based solely on FITC fluorescence. A small amount of overlap would be of no consequence if the two populations of interest existed in approximately equal proportions. However, leukocytes which are outnumbered approximately 1:100 in whole blood, would likely be obscured by the relatively immense erythrocyte distribution.

Erythrocyte-enriched samples were noted to autofluoresce greater than leukocyte-enriched samples in the red spectra as detected by FL-3 in unstained samples. Utilizing the inverted relationship of FL-1 and FL-3 in erythrocyte- and leukocyte-enriched samples stained with 25 μg/mL FITC and two-parameter analysis, superior differentiation of these populations was evident (Figure 2.6). Encompassing erythrocyte- and leukocyte-enriched samples in regions X and Z resulted in considerably less overlap of events. Even with this increased resolution, it was evident that based upon FITC-staining alone, an accurate total leukocyte count could not be performed. Additional findings suggested that damaged erythrocytes, or free erythroid nuclei, also demonstrated increased staining ability. Two findings supported this supposition. Erythrocytes being overwhelmingly predominant in whole blood and

erythrocyte-enriched samples, the higher staining peaks seen in these 0.3% NaCl sample FL-1 histograms were doubtlessly a subpopulation of these cells (Figure 2.8). This was also illustrated as increased numbers of events in region Z in hypotonic solutions (Figure 2.9). Therefore, calculation of leukocyte counts using this region could be artifactually inflated by any manipulation of blood samples increasing the number of damaged erythrocytes.

Other inconsistencies were detected that preclude the use of FITC as a leukocyte differentiating stain. Sorting whole blood stained with this fluorochrome revealed both erythrocytes and leukocytes on either side of the threshold. Erythrocytes falling to the right (higher fluorescence) could be eliminated by an additional two-parameter analysis on the cells remaining after ignoring events below the threshold. However, the numerous leukocytes present in each of the left sides (lower fluorescence) would be omitted by placing a minimum fluorescence threshold at any point in the whole blood cell distribution. This would induce an artifactually low total count.

Because of the difference in fluorescence noted in both fluorescent microscopy and FCM studies, these investigations examined the utility of staining unfixed, diluted chicken cells with FITC. Some authors suggest that the use of FITC requires prior fixation to allow intracellular access to this polar compound (11,52,121-124). Although the findings herein presented evidence that FITC stains unfixed leukocytes, the additional staining of damaged erythrocytes reflected this consensus. Additionally, FITC has been reported to bind variably to cytoplasmic protein even in a

homogeneous population of cells (122). This inconsistent staining could result in distribution variances not attributable to differences in cell type which may confuse interpretation.

The lability and decay of FITC- induced fluorescence presented another concern. The difference in dot plot appearance in different samples of the same cell type may also represent differences in the time from staining to analysis. Even with a discovery of more specific identification of leukocytes using FITC, e.g. by specific erythrolysis, this instability would require stringent reagent controls. Otherwise, the user would face the arduous task of establishing minimum fluorescence values, thresholds, and regions on each individual sample.

An additional issue when using a non-specific parameter to differentiate leukocytes from erythrocytes such as cytoplasmic protein is the potential inability to distinguish thrombocytes. Ideally, thrombocytes would be included in the erythrocyte peak, and thus excluded from the leukocyte population. Inclusion of thrombocytes in the leukocyte distribution would require their inclusion in the differential count. This may not be an undesirable calculation. Avian thrombocytes exhibit properties unlike mammalian platelets, such as phagocytic activity (148,171). Thus it may be more appropriate to include the thrombocytes in the total leukocyte count for non-mammalian species.

Two analogs of FITC, fluorescein diacetate (FDA) and carboxyfluorescein diacetate (COFDA), have been utilized in FCM to identify viable cells (37,172-175). These lipophilic compounds are non-fluorescent when unbound and freely movable

between the medium and cytoplasm. Intracellularly, they are converted to the fluorescent compounds fluorescein and carboxyfluorescein and accumulate because of their polarity, termed "fluorochromasia" (7,174). This conversion is reflective of the esterase activity of the cell; the enzyme kinetics and uniform fluorescence in cells suggest that multiple intracellular enzymes are responsible for the hydrolysis of these fluorochromes in mammalian tumor culture cells, including lipase, acylase and chymotrypsin (176).

Chicken lymphocytes exhibit α-naphthyl acetate esterase activity (111), although few cytochemical descriptions have noted the presence of other esterase activity in avian leukocytes. A difference in the presence of this enzyme in erythrocytes and leukocytes may permit recognition by fluorescence using these compounds. Additional reactive fluorochromes accumulating due to other enzyme activity in these cells also provides a source for future investigations.

Regardless, these studies indicated that a total leukocyte count based solely on cytoplasmic content utilizing FITC was not possible. Although fixation of whole blood may result in additional staining, the lability of fluorescence and the inconsistencies of staining will likely preclude its use as a rapid, economical, and convenient method that could be adapted to commercial hematology instrumentation.

Chapter 3: Flow Cytometric Differentiation of Avian Erythrocytes and Leukocytes with Thiazole Orange

Introduction

Considerable emphasis in veterinary medical research is placed on improving clinical diagnostics for non-mammalian species. One of the foundations for proper evaluation of any patient is a complete blood count, which includes a total leukocyte count or concentration. Currently, there are three manual methods in determining this parameter in non-mammalian species: direct, semi-indirect, and indirect. Direct enumerate leukocytes with specific stains and hemacytometers. The semi-indirect method stains only heterophils and eosinophils, which allows easier summation of positive cells in the hemocytometer. The total leukocyte number is then back-calculated using the differential count (96). The indirect methods estimate the number of leukocytes based on a comparison to erythrocytes on a blood smear.

There are many disadvantages to these manual methods in evaluating the total leukocyte concentration. The greatest source of error is the relatively small sample size (68). Although a notable degree of precision may be achieved if the cell counts are high (101), inherent error in using hemacytometers often overshadows any degree of accuracy (105). The most commonly utilized method is the semi-indirect (77), which relies on the differential count for calculation of the total leukocyte count. Slight deviations in the number of observed granulocytes from technician inexperience or an improper slide preparation can dramatically alter the calculated count (96,102).

The higher precision and accuracy available with automated hematology systems has resulted in essentially replacement of manual methods in diagnostic medicine (67). In addition, the costs of training and labor are reduced considerably with electronic systems (19,68,94). Unfortunately, the presence of the nucleus in the non-mammalian erythrocyte excludes the use of currently available blood cell analyzers in these species (102).

Flow cytometry (FCM) is a discipline which utilizes instruments to examine individual cells in suspension. Basically, a flow cytometer consists of a system of fluidics which separate a suspension of cells into a stream. This stream flows through an interrogation point, usually provided by a laser beam. Signals produced by this interrogation, such as light scatter, fluorescence, or absorbance are analyzed by computer interface (4). Since cells are examined individually, properties such as cellular size, density, and dye uptake can be evaluated on a particular population of cells within a heterogeneous sample (7). Their rapid analysis and high sensitivity has projected the use of FCM and related technology into the limelight of analytical cytometry (5).

Automated hematology instruments are based on the principles of FCM. In fact, some commercial units differ little in design and function from FCM instruments intended for basic research applications. These studies evaluated basic FCM techniques for their potential in performing total leukocyte counts in an avian species using a nucleic acid fluorochrome. The nucleus of mature avian erythrocytes contains highly compacted chromatin (126) and was therefore expected to stain with less

fluorescence intensity than other blood cells (46). Although mature chicken erythrocytes may contain pre-mRNA, the amounts are negligible when compared to actively transcribing cells (127). Therefore, RNA in leukocytes was expected to contribute to an increased staining ability.

The analysis of nucleic acid content was one of the earliest, and remains one of the most popular, uses of flow cytometric technology (60). Intercalating dyes such as ethidium bromide (EB) and propidium iodide (PI) have been utilized as nucleic acid dyes for DNA and cell-cycle analysis (122). The binding of these compounds to nucleic acids results in increased fluorescence quantum efficiency, which equates to increased florescence intensity (177). In high concentrations, EB and PI can also electrostatically bind to single-stranded nucleic acid (178-181). Propidium iodide in isotonic solutions is not incorporated into live cells; its exclusion has been used to indicate cell viability (182). In avian species, PI has been used to study DNA abnormalities in ducks (*Anas platyrhinchos*) exposed to environmental radiation contaminants (183).

A disadvantage of using EB and PI is their requirement of cell fixation (72,179,182). Nonetheless, intercalating dyes have been suggested for utilization in automated hematology. Shapiro *et al.* described the use of EB, brilliant sulfaflavine (BSF), and a stilbene disulfonic acid derivative dye known as LN ("long name"), to identify the five leukocyte subpopulations in human blood (72). Both an argon and a helium-cadmium laser for scatter and absorption measurements were necessary for this differentiation.

Another fluorochrome which has been used in hematologic systems is the metachromatic dye acridine orange (AO) (120,184). This dye has the additional ability to differentiate single-stranded and double-stranded nucleic acids. As an intercalating dye staining double-stranded nucleic acids, AO emits light in the green spectrum (approximately 530 nm); as a single-stranded stain, dye-to-dye interactions result in fluorescence in the red spectra (640 nm) (117). Double-stranded RNA can be selectively denatured, such as the presence of chelating agents, to ensure that green fluorescence represents DNA only for cell cycle analysis (116). Leukocyte granules also fluoresce red when stained with AO, which permits the identification of leukocytes into three categories: lymphocytes, monocytes, and granulocytes (73,118,119). There are several disadvantages to using AO. Probably the most important is the strict attention to stain concentrations required; compensation for loss of stain in the sheath fluid or to adsorption onto glass and plastic surfaces may be necessary. This adherence to surfaces within the FCM also necessitates extensive cleaning steps to prevent interference in subsequent analyses (117).

Thiazole orange (TO) was recently described by Lee *et al.* as a potential reticulocyte dye (125). Unlike other dyes suggested for this purpose, such as pyronin Y, $DiOC_1$, acridine orange, and thioflavin T, TO does not require laborious staining procedures or extensive data manipulation. This compound was found to have many desirable characteristics, namely, increased fluorescence when bound, membrane permeability, and superior excitation at 488 nm, which is compatible with the argon ion laser found in most FCM instruments. The increased fluorescence of the dye is

contributed to the presence of endocyclic nitrogen and planar restriction when bound. Interestingly, this report referred to an identification of human leukocytes by a high degree of fluorescence. Since TO binds to both single- and double-stranded nucleic acid, this intensity was most likely a result of the DNA present in these cells. The use of TO for enumerating reticulocytes has been favorable in humans, dogs, and cats (155,185,186).

This series of experiments investigated the use of TO to preferentially stain chicken leukocytes and thrombocytes. This preference was then utilized to differentiate and quantify these populations using standard flow cytometric techniques. Blood cells were divided into three samples (whole blood, erythrocyte-, and leukocyte-enriched) and compared. Populations identified in FCM-generated graphical displays were numerically compared with manual cell counts. Cells were also subjected to various hypotonic solutions to determine the effects of cell damage on TO staining.

Materials and Methods

Blood collection

Adult white leghorn hens from the Louisiana State Agricultural Experimental Station, Central Station - Poultry Unit were used as blood donors. Approximately 5 mL of blood was drawn from the cutaneous ulnar vein into K_2EDTA coated syringes with 25 gauge needles. The blood was then immediately transferred to K_3EDTA collection tubes (Vacutainer®, Becton Dickinson Vacutainer Systems, Rutherford, NJ).

Blood separation

Twenty μL of whole blood from two chickens was set aside for the whole blood samples. The remainder was centrifuged at 200 *g* for 10 minutes (Beckman® Model YJ-6 centrifuge, Beckman, Palo Alto, CA). Cells were removed from the erythrocyte pellet for erythrocyte-enriched samples. The plasma, buffy coat, and upper layer of the erythrocyte pellet were placed in 1.0 mL hematocrit tubes (Seditube™, Becton-Dickinson, Rutherford, NJ) and centrifuged at 200 *g* for 10 minutes. The resulting plasma and buffy coat was then removed as the leukocyte-enriched sample. The erythrocyte- and leukocyte-enriched samples were diluted in phosphate-buffered saline (PBS) in a 1:1 ratio to approximate a 50% packed cell volume.

Hypotonic lysis

Fifty μL of each separated sample (whole blood, erythrocyte-, and leukocyte-enriched) from two chickens were added to 0.5 mL of each of four concentrations of saline (0.2%, 0.4%, 0.6%, and 0.9%). These were allowed to incubate at room temperature (RT) for approximately 30 minutes.

Stain preparation and use

A 0.1 mg/mL stock solution of thiazole orange (Becton Dickinson Immunocytometry Systems, San Jose, CA) was prepared in ethanol and stored at -10° C. A working solution of 0.1 μg/mL was prepared in PBS just prior to use. For all trials, blood cell samples were suspended in this working solution for a final dilution

of 1:200 and incubated approximately 1 hour as described by Lee, *et al* (125). Five μL of each sample in 1.0 mL PBS served as controls.

Manual leukocyte counts

Total leukocyte counts were performed on six chickens (identified as A-F) using the semi-indirect method described by Wiseman (103). Whole blood was diluted and stained with phloxine (Unopette® Test 5877, Becton-Dickinson Vacutainer Division, Rutherford, NJ), which stains heterophils and eosinophils. These cells were counted by light microscopy in a hemacytometer with Neubauer ruling. Differential counts were performed on blood smears stained using a modified-Wright's stain (Hema-tek® Model 4480, Ames Company, Division of Miles Laboratories, Elkhart, IN). One hundred cell differential counts of leukocytes and 500 cell differentials that included both leukocytes and thrombocytes were performed. The total leukocyte or combined leukocyte/thrombocyte count (cells/μL) was then derived with the relative number of heterophils and eosinophils in the differential count and the absolute number derived from the hemacytometer using the equation (96):

$$\frac{(\text{\# stained cells})(1.1)(16)}{(\%\ \text{heterophils} + \text{eosinophils in differential})/100}$$

Instrument- and hemacytometer-derived total cell counts were also performed. In Experiment I, a total nucleated cell count was derived from the erythrocyte channel of a commercial electronic cell counter (Baker System 9000 Automated Cell Counter, Baker Instruments Corporation, Allentown, PA). In Experiment II, a Neubauer-ruled hemacytometer was charged with a 1:200 dilution of whole blood in PBS. A total cell

count (cells/μL) was derived by counting all cells in thirteen of the 25 small central squares and using the following equation:

$$\frac{(\#\ \text{cells})(200)}{(13)(0.004\ \mu L)}$$

Flow cytometry

Flow cytometric analysis of the separated blood samples was performed with a commercial instrument (FACScan™, Becton Dickinson Immunocytometry Systems, Becton, Dickinson, and Co., San Jose, CA). This unit used a 15 milliwatt, air-cooled, 488 nm argon ion laser and sterile physiologic saline as sheath fluid. Detection was supplied by photomultiplier tubes as: FL-1 (green fluorescence), FL-2 (orange fluorescence), FL- 3 (red fluorescence), SSC (side-scatter), and FSC (forward scatter). Fluorescence detectors were amplified logarithmically and incorporated a 530/30 nm and 585/42 nm band pass filter for green and orange, and a 670 nm long pass filter for red fluorescence. Data was acquired in list mode to a computer interface (Hewlett-Packard HP 9000 series, model 340, San Diego, CA) and analyzed using supplied software. Fluorescence detectors were set at 400 (FL-1), 400 (FL-2), and 654 (FL-3). On separated and lysed samples, 10,000 cells were collected. In the remaining trials, over 200,000 cells were collected and tabulated.

Following FCM analysis, a slide was prepared for each sample using a cytocentrifuge (Cytospin®, Shandon Southern, Sewickley, PA), stained with a modified-Wright's stain, and examined.

Data analysis

Histograms and dot-plots in various combinations were produced for each parameter measured (FSC, SSC, FL-1, FL-2, and FL-3) for each sample and visually compared. Several polygonal regions (X, Y, Z, and Q) were created using analytical software (Lysys™ II, Becton-Dickinson Immunocytometry systems, San Jose, CA) for comparison of samples using the SSC vs. FL-2 dot plot; the number of events in each region was also computed. Another analytical software package (Paint-a-Gate™, Becton-Dickinson Immunocytometry Systems, San Jose, CA) was used to identify populations of interest and follow their position in displays of other parameters. This program enables the user to color areas of a dot-plot or histogram in one graph and trace that population in other graphs. For example, a small population of high intensity staining cells seen in the SSC vs. FL-2 dot plot (or the FL-2 histogram) can be visualized in a FSC vs. SSC dot-plot.

The relative proportions of these regions (expressed as a percentage of all cells analyzed) were converted to numerical amounts by multiplying their respective percentages by total cell counts per unit volume. In Experiment II, counts of events in regions Y and combined Y + Z were also calculated using a timed analysis. The number of events occurring in each region was recorded for a 4 minute analysis at a rate of 60 μL/minute. This value represents the total number of cells in 240 μL of the 1:200 sample. Therefore, total cells per μL were calculated by dividing this number of events by 240 and multiplying by 200. For these experiments, the threshold for FCM analysis was set using the FL-1 channel at approximately 672 units. This

effectively ignored all events that exhibited low fluorescence, i.e. the majority of those falling in the X regions and all in Q. In this manner, the relative number of cells in the Y and Z regions was increased. This served to increase the sample size of cells evaluated in these two regions.

Based on the observations made from the distributions of events in the FL-2, FSC, and SSC displays, cells within regions X, Y, and Z were compared to erythrocytes, thrombocytes, and leukocytes, respectively. Counts of cells in region Z were compared to manual leukocyte counts, and in regions Y + Z with combined leukocyte/thrombocyte counts using a linear regression model.

Results

Separated blood

Frequency histograms of FL-2 fluorescence and FSC vs. SSC dot plots for whole blood, erythrocyte-, and leukocyte-enriched samples are presented in Figures 3.1, 3.2, and 3.3. The whole blood and erythrocyte-enriched samples were virtually identical in both stained and control samples. This was expected, as erythrocytes are by far the most numerous cell in whole blood. The leukocyte-enriched samples, however, contained different populations in fluorescence, FSC, and SSC, when compared to the other two samples. In the FL-2 frequency histogram, the fluorescence peaks near channel 200 (indicated by marker M1) in the leukocyte-enriched samples corresponded to the a smaller peak seen in the erythrocyte-enriched and whole blood samples.

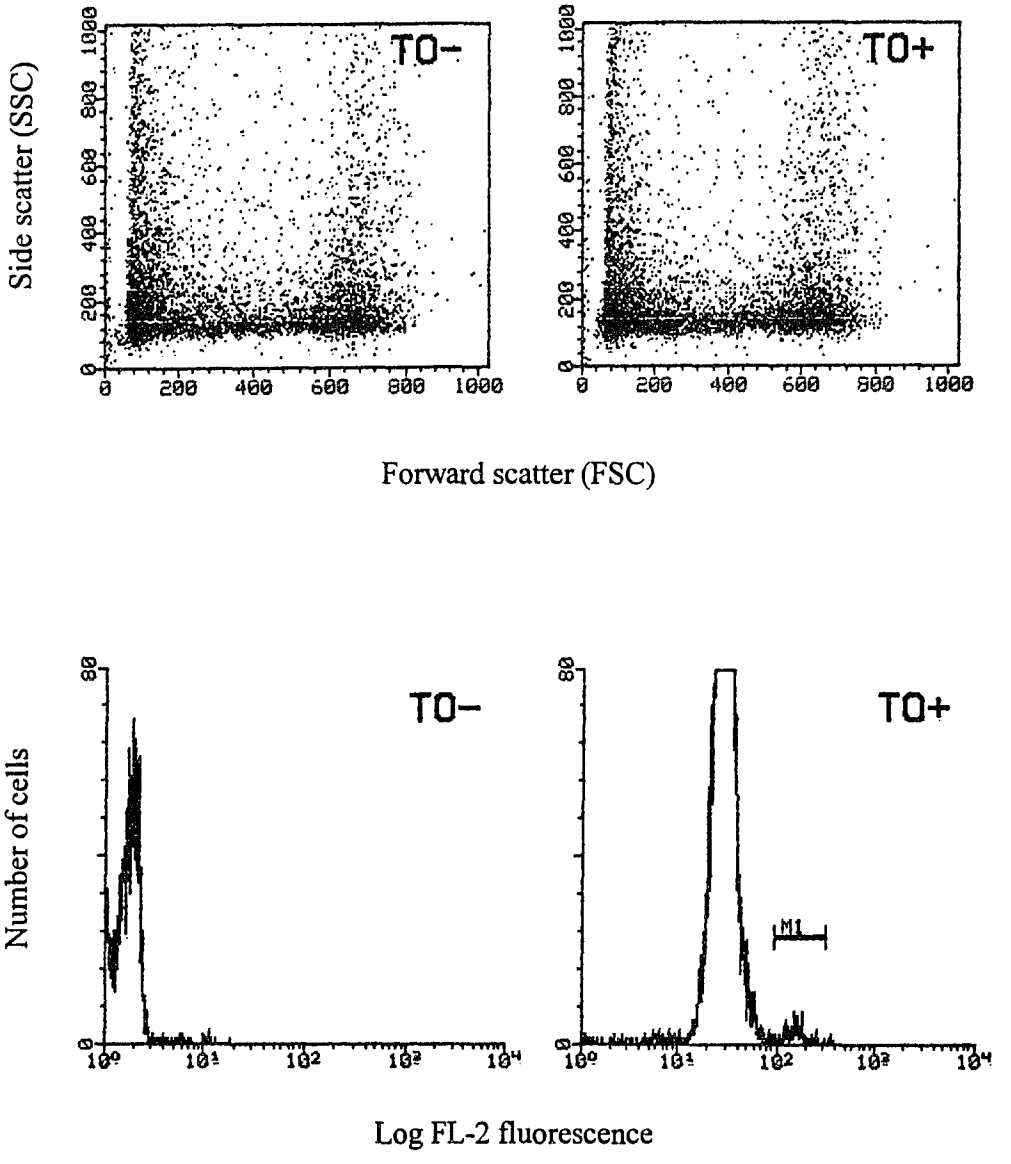

Figure 3.1. FL-2 frequency histograms and FSC vs. SSC dot plots of whole chicken blood samples unstained and stained with TO. Note identification of peak channel of the small, higher-intensity staining population (M1) (compare with Figures 3.2 and 3.3).

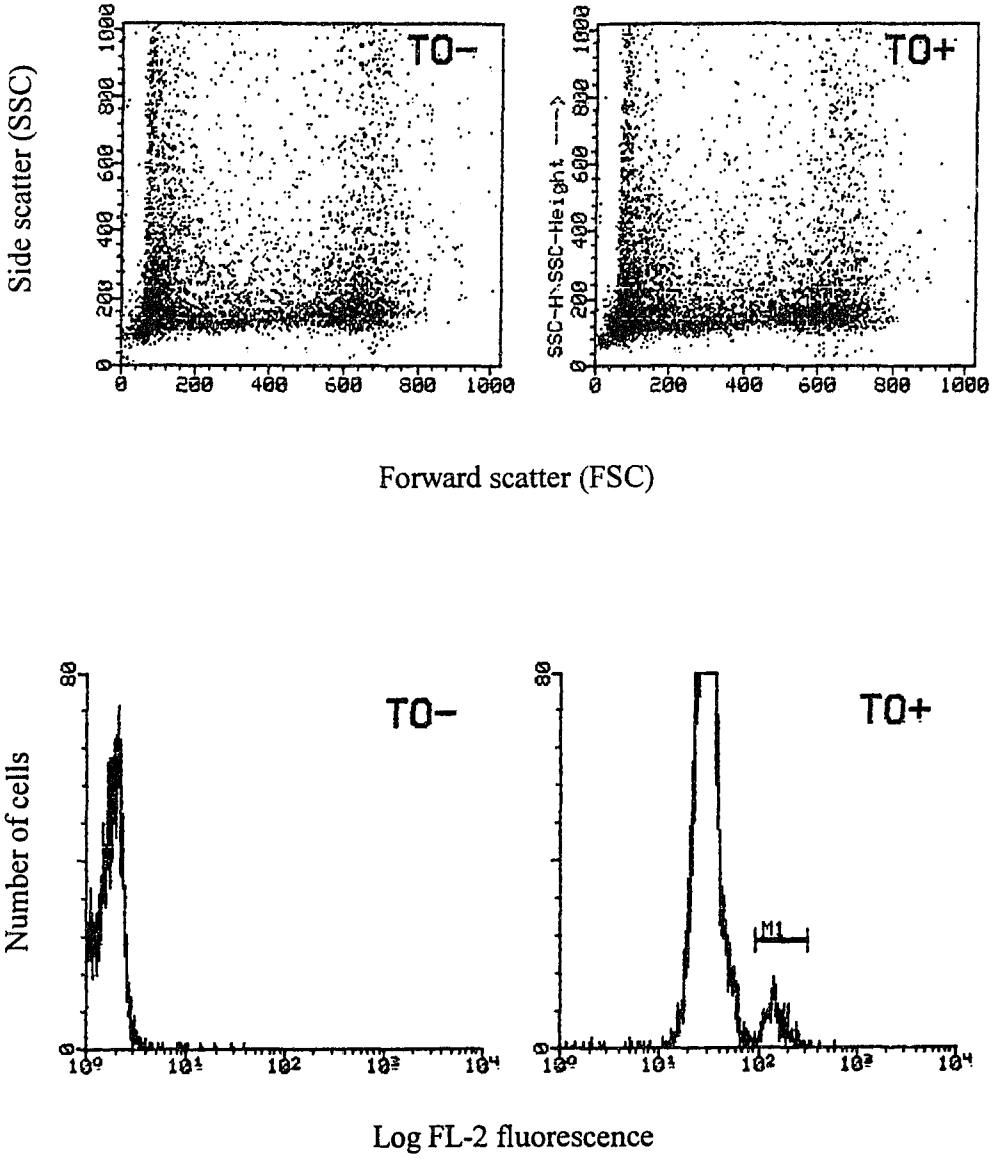

Figure 3.2. FL-2 frequency histograms and FSC vs. SSC dot plots of erythrocyte-enriched chicken blood samples unstained and stained with TO. Note identification of peak channel of the small, higher-intensity staining population (M1) (compare with Figures 3.1 and 3.3).

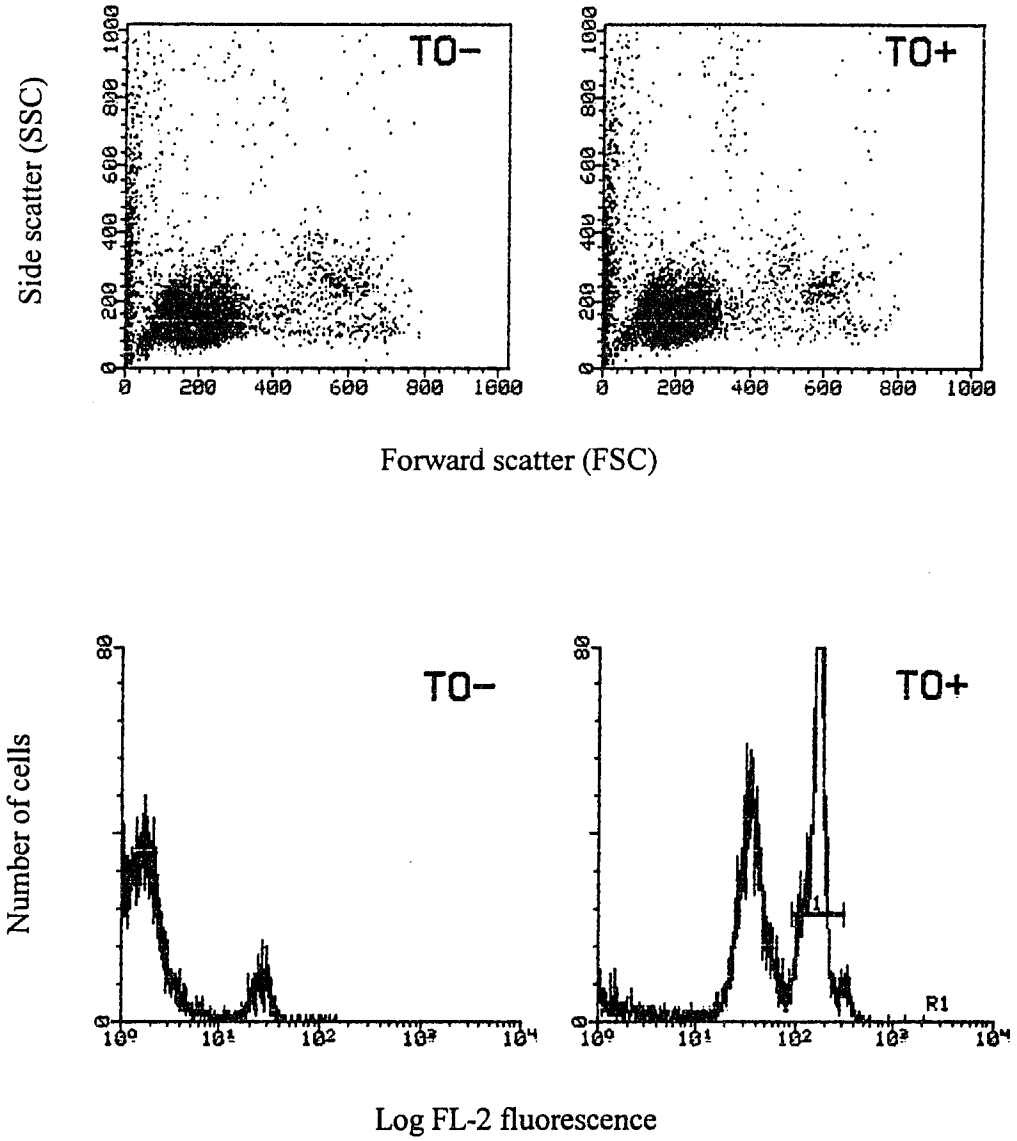

Figure 3.3. FL-2 frequency histograms and FSC vs. SSC dot plots of leukocyte-enriched chicken blood samples unstained and stained with TO. Note identification of peak channel of the small, higher-intensity staining population (M1) (compare with Figures 3.1 and 3.2).

Examination of the samples using a two-parameter analysis of SSC vs. FL-2 provided better differentiation of multiple populations (Figure 3.4). The largest population of events in whole blood, erythrocyte-enriched and to a lesser extent in leukocyte-enriched samples was encompassed by the region X. Regions Y and Z were drawn to enclose the higher-intensity stained populations seen in the leukocyte-enriched samples, corresponding to smaller populations in the erythrocyte-enriched and whole blood samples. In addition, the leukocyte-enriched samples contained a large amount of low-intensity staining cells, corresponding to a smaller population in the whole blood and erythrocyte-enriched samples. Region Q was created to circumscribe these events.

Hypotonically lysed blood

Dot plots of SSC vs. FL-2 for whole blood, erythrocyte-, and leukocyte-enriched sample diluted in 0.2%, 0.4%, 0.6%, and 0.9% NaCl are presented in Figures 3.5, 3.6, and 3.7. To facilitate analysis, the number of events in the regions X, Y, Z, and Q was calculated in whole blood and leukocyte-enriched samples. Results of these findings are presented in Table 3.1 and Figure 3.8. These numerical values represent the means of two samples performed at each concentration for each cell type. In both the whole blood and leukocyte-enriched samples, the relative amounts of cells in region X increased as the diluent approached isotonicity, i.e., decreased with additional lysis. Conversely, the proportion of the combined cells in regions Y and Z tended to increase in lower NaCl concentrations. Another difference in the sample types was the proportion of cells in region Q, which remained fairly constant

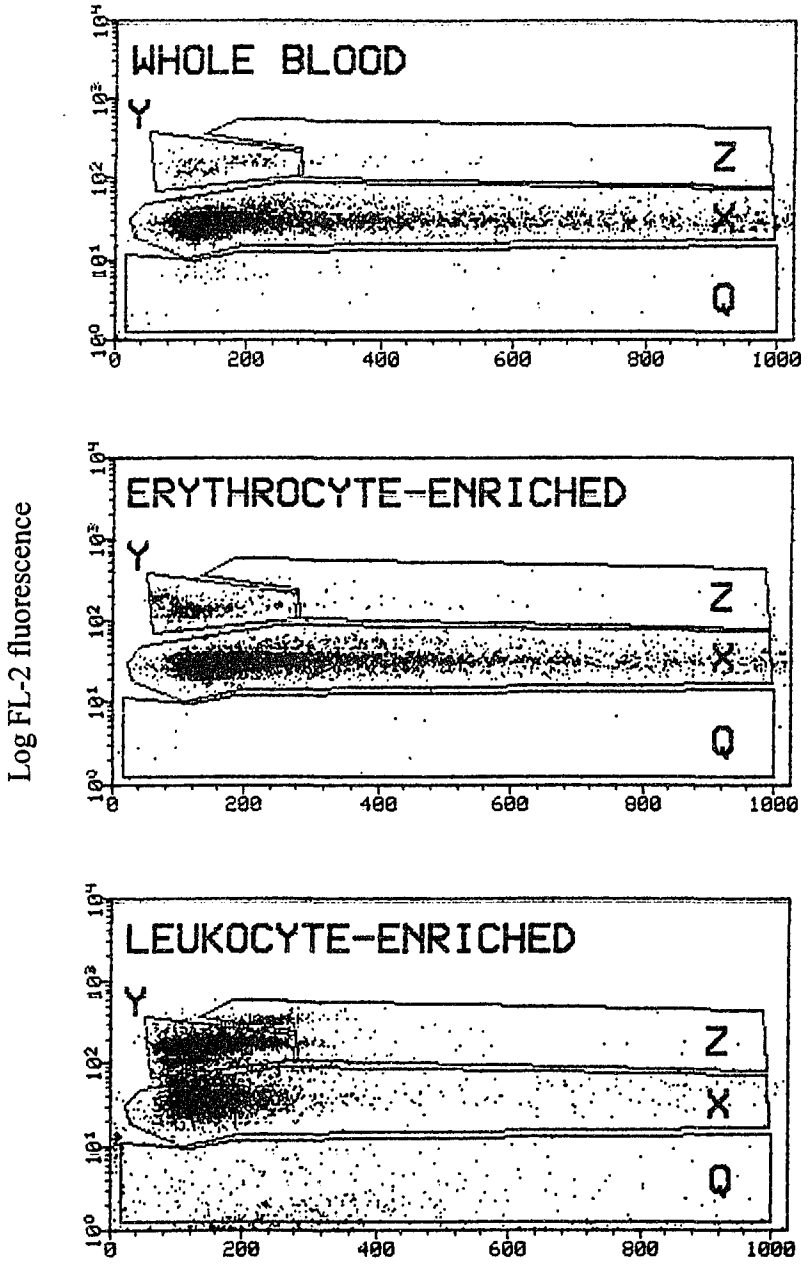

Figure 3.4. SSC vs. FL-2 dot plots of whole blood, erythrocyte-, and leukocyte-enriched samples stained with TO demonstrating regions Q, X, Y, and Z. Regions Y and Z were drawn to encompass the higher fluorescence intensity populations seen in leukocyte-enriched sample; region X the large population in erythrocyte-enriched sample; region Q the remaining events.

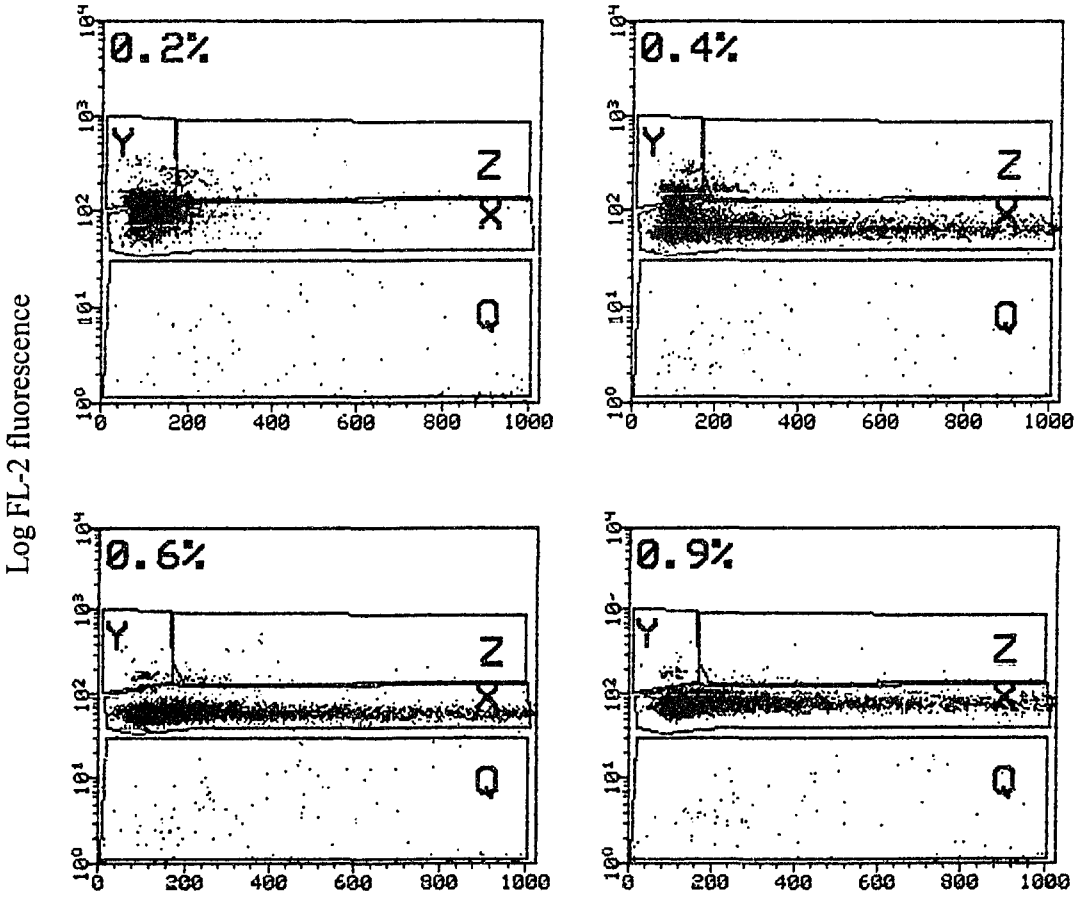

Figure 3.5. SSC vs. FL-2 dot plots for whole blood samples diluted in 0.2%, 0.4%, 0.6%, and 0.9% NaCl stained with TO. Note the relative increase of events in the Y and Z regions in hypotonic solutions.

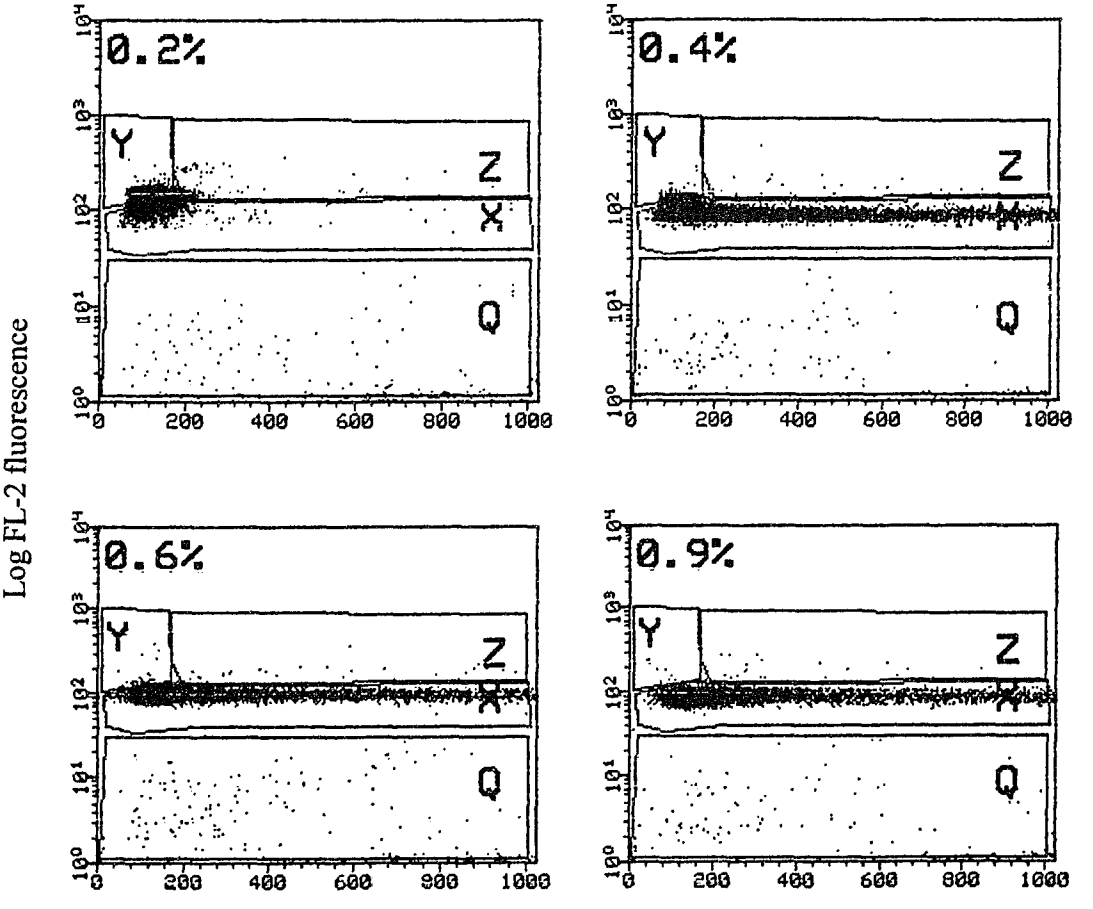

Figure 3.6. SSC vs. FL-2 dot plots for erythrocyte-enriched samples diluted in 0.2%, 0.4%, 0.6%, and 0.9% NaCl stained with TO. Note the relative increase of events in the Y and Z regions in hypotonic solutions.

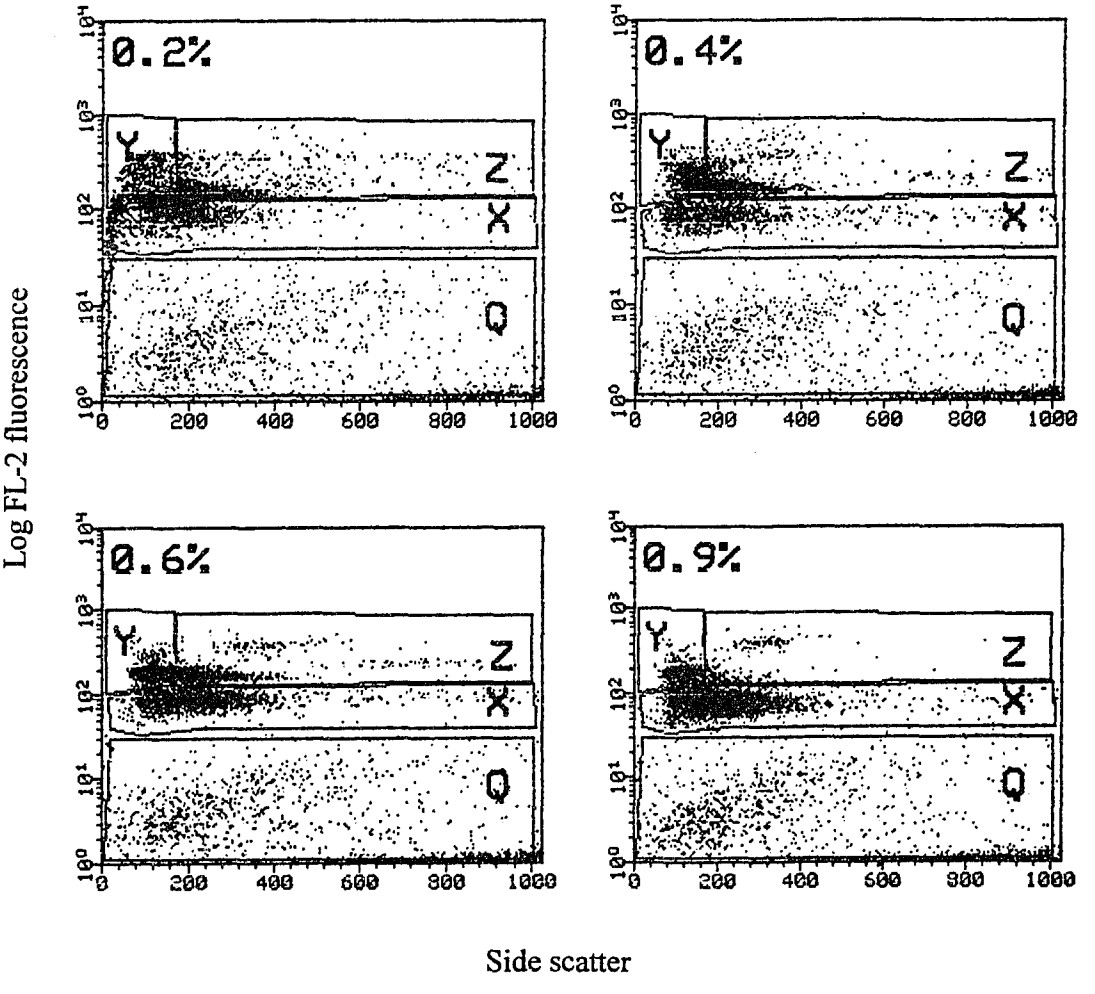

Figure 3.7. SSC vs. FL-2 dot plots for leukocyte-enriched samples diluted in 0.2%, 0.4%, 0.6%, and 0.9% NaCl stained with TO. Note the relative increase of events in the Y and Z regions in hypotonic solutions.

Table 3.1. Mean number of cells in regions X, Y+Z, and Q in whole blood and leukocyte-enriched samples diluted in 0.2%, 0.4%, 0.6%, or 0.9% NaCl

	0.2%	**0.4%**	**0.6%**	**0.9%**
X in LE	1327	1291	1855	2831
Y+Z in LE	1935	2556	1882	1034
Q in LE	6737	6153	6262	6134
X in WB	3124	5924	6449	6787
Y+Z in WB	5629	602	92	66
Q in WB	1246	3473	3458	3146

Note that each value represents the number of events in each region in a total of 10,000 total analyzed.
LE = leukocyte-enriched; WB = whole blood

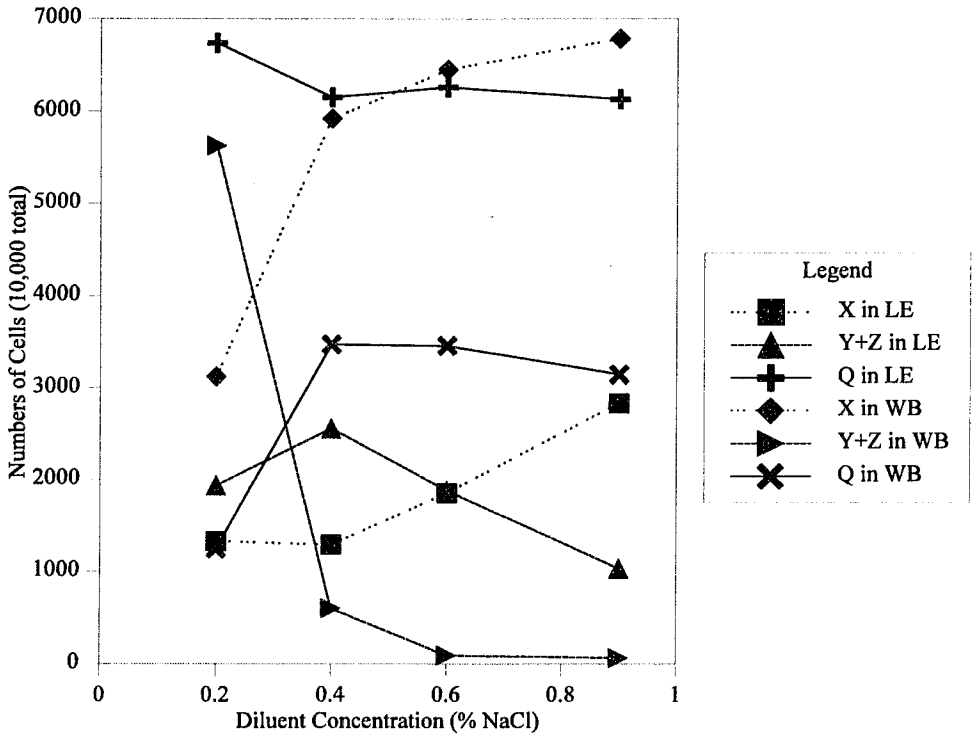

Figure 3.8. Mean number of cells in regions X, Y+Z, and Q in whole blood and leukocyte-enriched samples diluted in 0.2%, 0.4%, 0.6%, or 0.9% NaCl. Each value represents the number of events in each region in a total of 10,000 cells analyzed. LE = leukocyte-enriched samples; WB = whole blood samples.

in leukocyte-enriched samples but decreased in whole blood samples in hypotonic solutions.

In another analysis, FL-2 histograms for erythrocyte- and leukocyte-enriched samples were compared. Gates were constructed to encompass the single large peak in the erythrocyte-enriched samples, and the first large peak in leukocyte-enriched samples indicated by M1 in 0.2% and M2 in 0.9% samples (Figure 3.9). The peak fluorescence channel for these peaks was determined and compared. These results are presented in Table 3.2. The average peak fluorescence channel for M1 in the 0.2% samples is significantly greater than the peak fluorescence channel for M2 in 0.9% samples ($p = 0.0124$). This indicates that cells in hypotonic solutions (0.2% NaCl) fluoresced to a greater extent than those in isotonic solutions (0.9% NaCl).

Events in regions X, Y, Z, and Q were identified using the coloring software (Paint-a-Gate™) and traced through several other displays (Figures 3.10, 3.11, and 3.12). In all samples, cells encompassed by the regions Q were distributed in the low FSC, variable SSC region (Figures 3.10, 3.11).

In whole blood samples diluted in 0.2% NaCl (Figure 3.10), events in region X were found throughout the SSC spectrum and in the central FSC regions. This was similar to most of the events in X in other diluents which are also found throughout the FSC spectrum with a high number in the high FSC. Events in region Y were found primarily in the low FSC/SSC region, with smaller numbers scattered in the low SSC, variable FSC. Events in region Z in 0.2% NaCl were found throughout the FSC vs. SSC dot-plot.

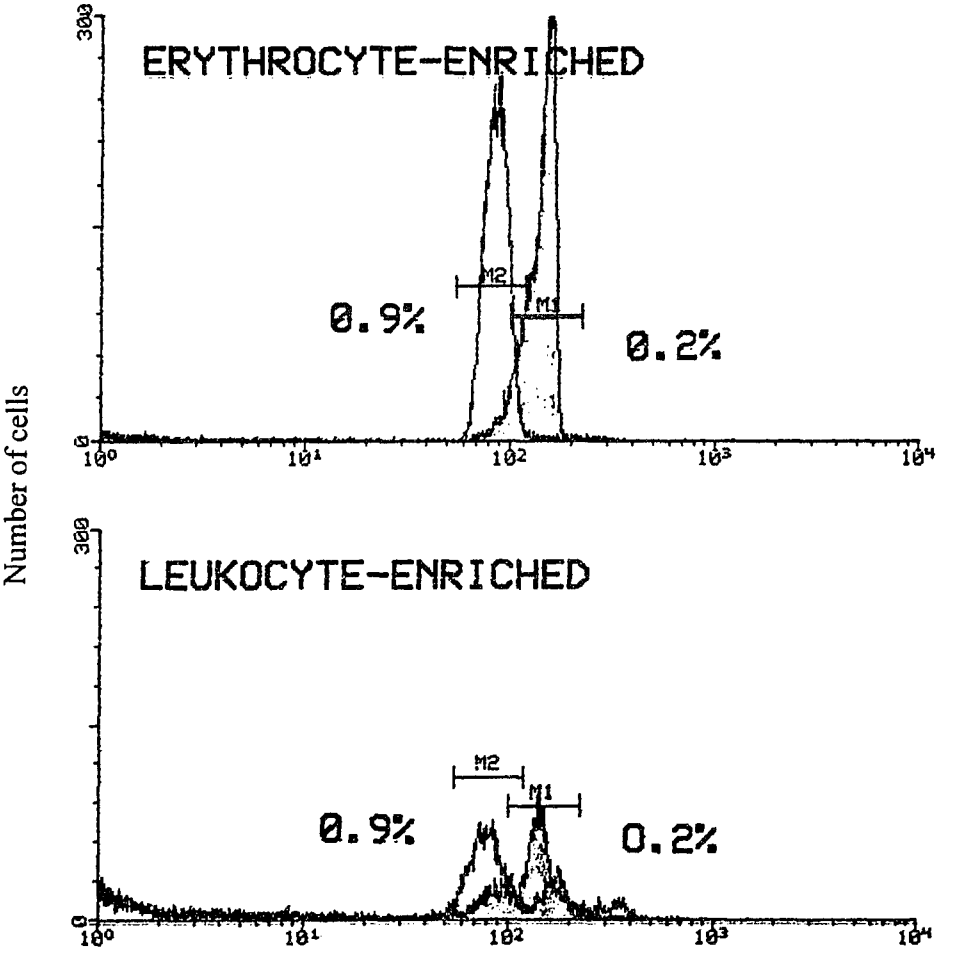

Figure 3.9. FL-2 fluorescence histograms of erythrocyte- and leukocyte-enriched samples in 0.2% and 0.9% NaCl demonstrating placement of gates for peak channel fluorescence measurement. Note that M1 was set to identify the primary peak in 0.2% samples, and M2 in 0.9% samples.

Table 3.2. Comparison of peak channel values in erythrocyte- and leukocyte-enriched chicken blood samples diluted in either 0.2% or 0.9% NaCl

Diluent concentration	Sample	Erythrocyte-enriched	Leukocyte-enriched	Mean ($\bar{X}$)
0.2% (M1)	A	121.88	94.75	125.65
	B	152.61	133.35	
0.9% (M2)	A	74.32	80.58	82.43
	B	89.77	85.05	

Note: Peak channel values are based on gates surrounding the primary peak seen in histograms of FL-2 fluorescence for each of these samples diluted either 0.2% (M2) or 0.9% (M1) NaCl (see Figure 3.9). For a one-tailed T-test, p value for difference in means equals 0.0124.

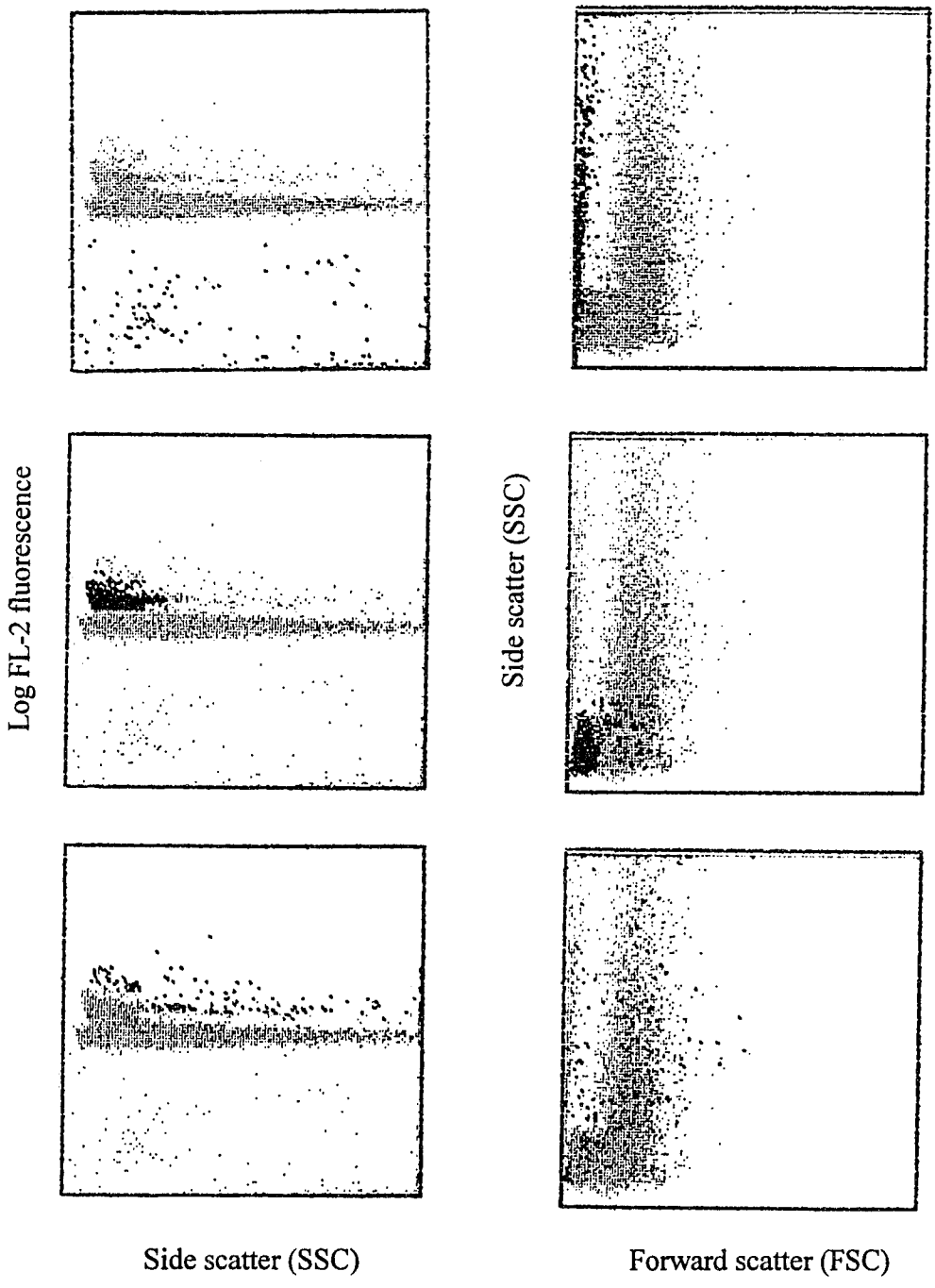

Figure 3.10. Color analysis ("Paint-a-Gate") of chicken whole blood cells in 0.2% NaCl stained with TO. Note the relative positioning of cells in regions Q (top), Y (middle), and Z (bottom) in the FSC vs. SSC and SSC vs. FL-2 dot plots indicated by the bold-faced dots.

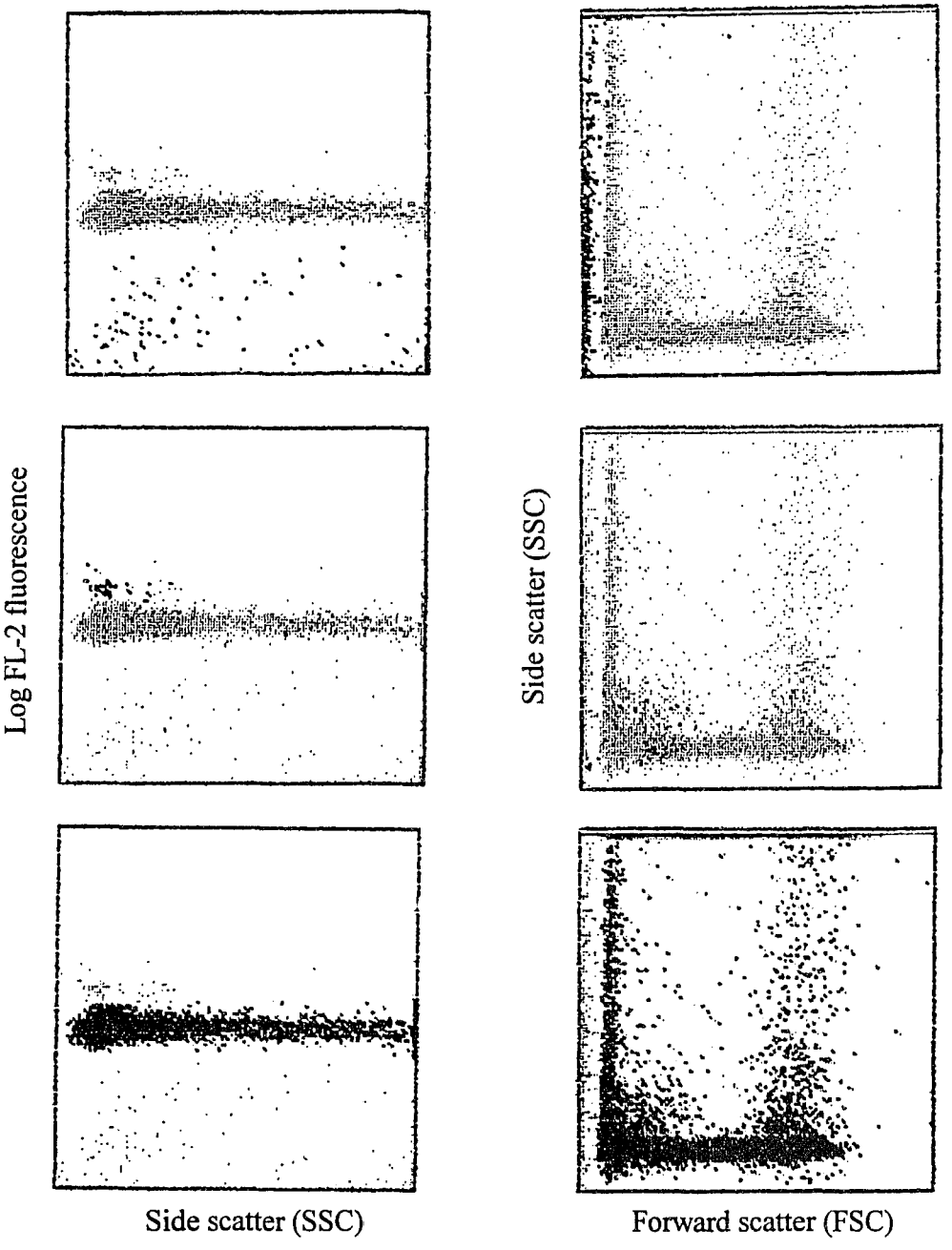

Figure 3.11. Color analysis ("Paint-a-Gate") of chicken whole blood cells in 0.9% NaCl stained with TO. Note the relative positioning of cells in regions Q (top), Y (middle), and X (bottom) in the FSC vs. SSC and SSC vs. FL-2 dot plots indicated by the bold-faced dots.

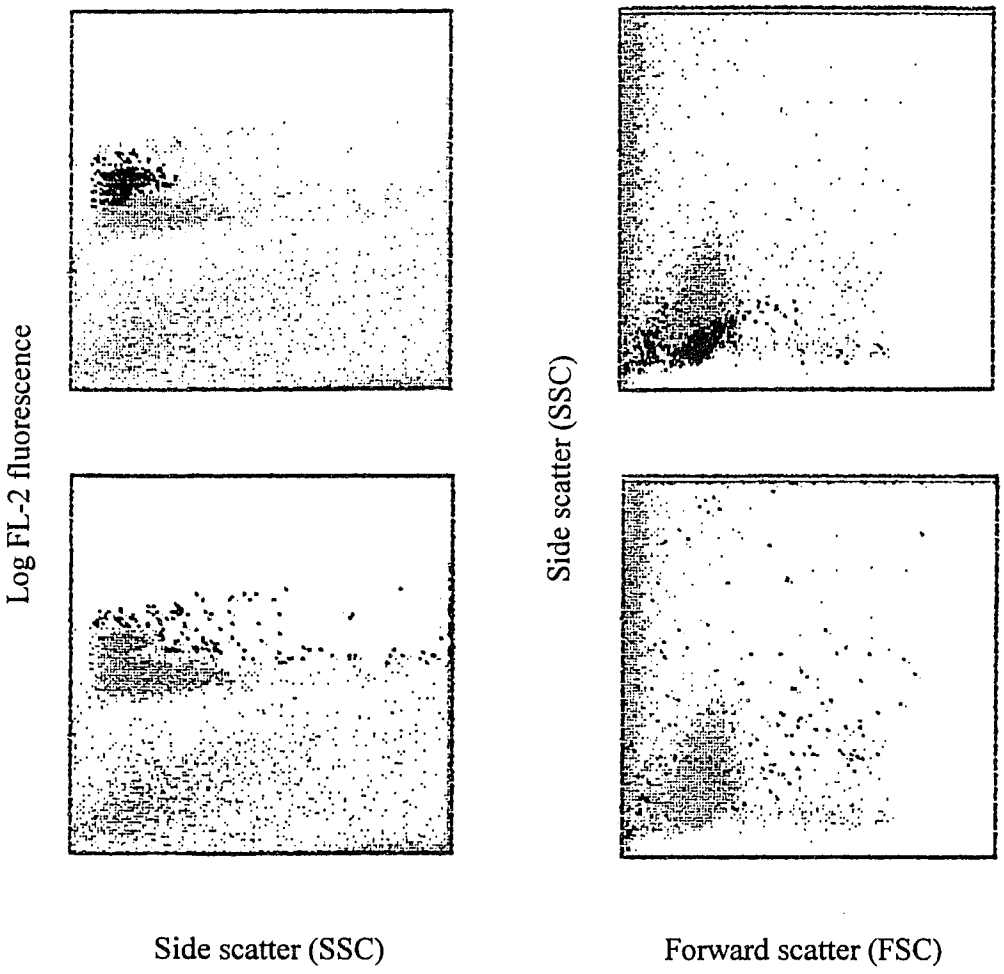

Figure 3.12. Color analysis ("Paint-a-Gate") of chicken leukocyte-enriched blood samples in 0.9% NaCl stained with TO. Note the relative positioning of cells in regions Y (top) and Z (bottom) in the FSC vs. SSC and SSC vs. FL-2 dot plots indicated by the bold-faced dots.

In whole blood diluted in higher concentrations of NaCl (Figure 3.11), events in X were distributed throughout the FSC/SSC spectra, with variable SSC primarily seen in the low and high FSC areas, separated by a line of low SSC, variable FSC. Events in region Y were present in low amounts in the low FSC/SSC region with more in the low SSC/moderate FSC region. This distinction was readily apparent in leukocyte-enriched samples diluted in 0.9% NaCl (Figure 3.12), in which the majority of this population was found at the higher FSC border of the X region. As in other samples, events in region Z were distributed throughout the FSC/SSC dot-plots.

Evaluation of total leukocyte counts

The results from Experiment I, in which the total cell count was calculated by an electronic cell counter (Baker System 9000) are presented in Table 3.3 and graphically displayed in Figures 3.13 and 3.14. There was no linear relationship evident when comparing the cell counts of region Z and the manual leukocyte count ($r^2 = 0.166$, slope = -1.25) or of the combined regions Y and Z and the manual combined leukocyte/thrombocyte counts ($r^2 = 0.834$, slope = -2.37).

In Experiment II, the total cell count was calculated using the hemacytometer method. Also in this experiment, counts of events in regions Y and Z were calculated by a timed analysis. Although the use of FL-2 fluorescence as a collection threshold increased the numbers of cells in Y and Z examined, it also resulted in obscuring the differentiation between the regions (Figure 3.15). These results are presented in Table 3.4 and graphically in Figures 3.16, 3.17, and 3.18. No linear relationship was evident when comparing the cell counts of region Z and the manual leukocyte count, using

Table 3.3. Cell counts by manual and FCM methods (Experiment I)

	Chicken Identification					
	A	**B**	**C**	**D**	**E**	**F**
Cells in region X (cells x 10^6)	0.318	0.289	0.328	0.275	0.280	0.277
Cells in region Z (cells)	631	815	846	577	858	309
Cells in regions Y + Z (cells x 10^3)	2.91	3.55	3.05	2.27	3.52	1.51
Total cells analyzed (cells x 10^6)	0.344	0.318	0.361	0.298	0.306	0.296
Total cell count (cells x 10^6/μL)	3.52	3.09	2.69	2.95	2.75	3.11
Region X count (cells x 10^6/μL)	3.25	2.81	2.45	2.72	2.51	2.90
Region Z count (cells x 10^3/μL)	6.46	7.93	6.31	5.71	7.71	3.24
Region Y + Z count (cells x 10^3/μL)	29.8	34.5	22.7	22.4	31.7	15.8
Manual leukocyte count (cells x 10^3/μL)	8.38	17.1	15.9	16.7	20.3	24.0
Manual combined leuckotye and thrombocyte count (cells x 10^3/μL)	69.8	55.4	79.6	97.6	77.3	105

Note: FCM-generated counts were obtained by multiplying the relative amounts of cells stained with TO in a given region (cells per region/total analyzed) by the total cell count, as obtained by the red cell channel of an electronic cell counter (Baker System 9000 Automated Cell Counter, Baker Instruments Corporation, Allentown, PA)

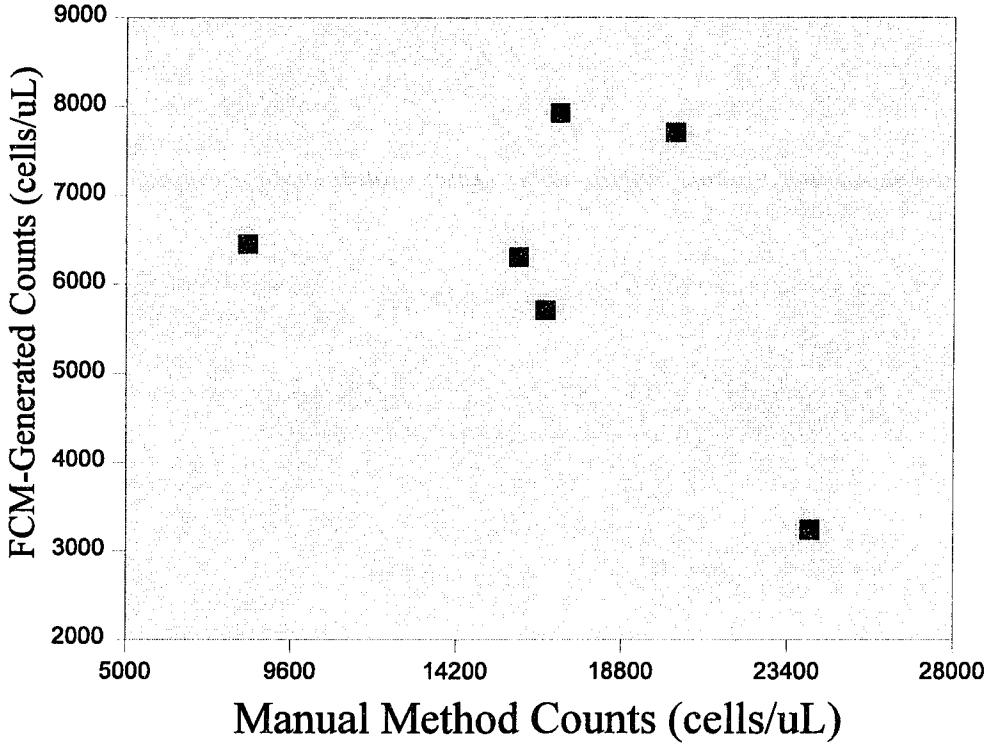

Figure 3.13. Scattergram comparison of total leukocyte counts: manual method vs. FCM-generated (Experiment I) using TO. FCM-generated counts were obtained by multiplying the relative amounts of cells in region Z (cells per region/total analyzed) by the total cell count, as obtained by the red cell channel of an electronic cell counter (Baker System 9000 Automated Cell Counter, Baker Instruments Corporation, Allentown, PA).

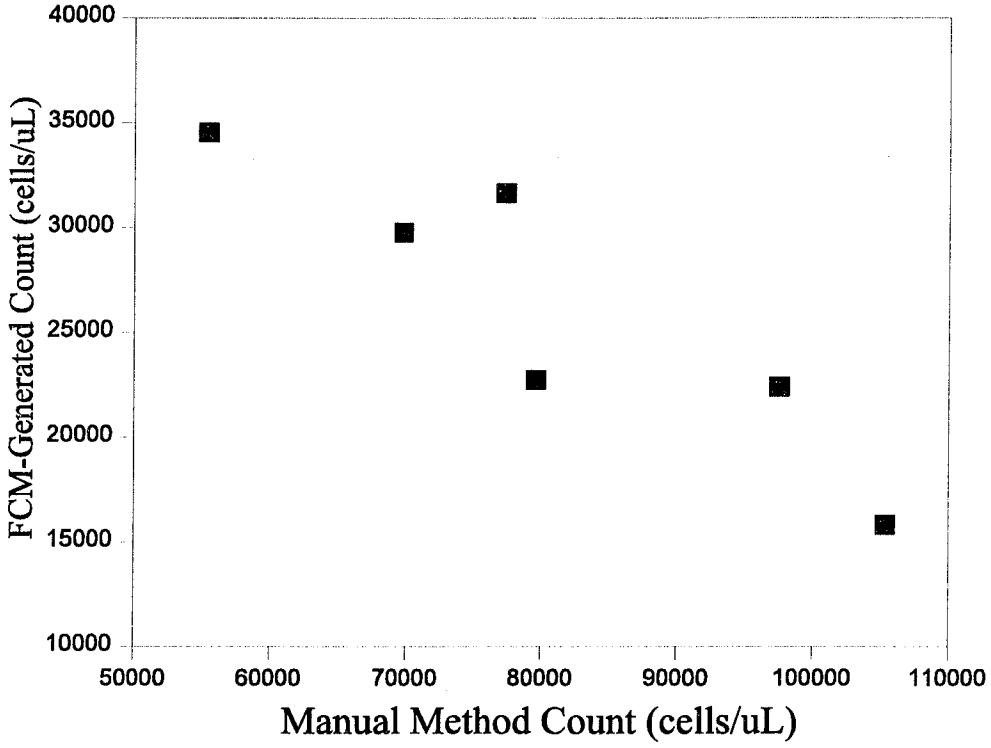

Figure 3.14. Scattergram comparison of combined leukocyte and thrombocyte counts: manual methods vs. FCM-generated (Experiment I) using TO. FCM-generated counts were obtained by multiplying the relative amounts of cells in regions Y+Z (cells per region/total analyzed) by the total cell count, as obtained by the red cell channel of an electronic cell counter (Baker System 9000 Automated Cell Counter, Baker Instruments Corporation, Allentown, PA)

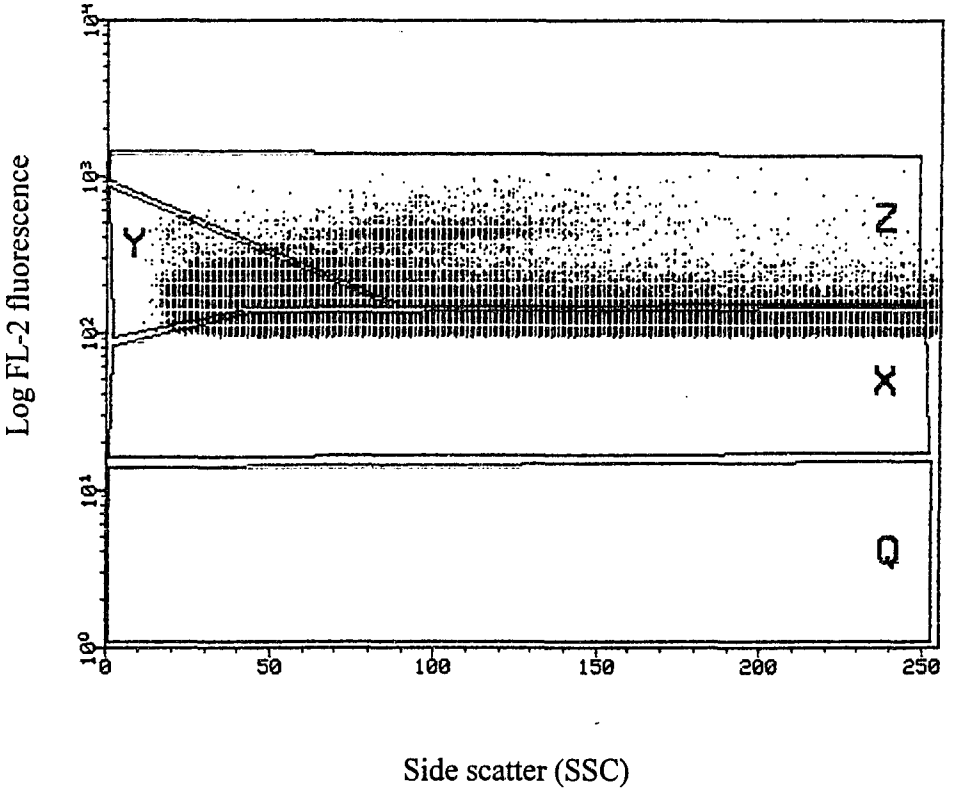

Figure 3.15. Representative dot plot of SSC vs. FL-2 using a minimum FL-2 fluorescence threshold to ignore all events in region Q and most of those in region X in whole blood stained with TO. Note the obscuration of delineation between regions X, Y, and Z.

Table 3.4. Cell Counts by manual and FCM Methods (Experiment II)

	Chicken Identification					
	A	**B**	**C**	**D**	**E**	**F**
Cells in region X (cells x 10^6)	0.289	0.287	0.287	0.287	0.287	0.289
Cells in region Z (cells)	176	263	272	183	327	173
Cells in regions Y + Z (cells x 10^3)	0.994	1.28	2.00	2.09	1.71	1.23
Total cells analyzed (cells x 10^6)	0.300	0.300	0.300	0.300	0.300	0.300
Total cell count (cells x 10^6/μL)	2.66	3.41	2.97	2.75	2.35	2.57
Region X count (cells x 10^6/μL)	2.56	3.26	2.85	2.64	2.25	2.47
Region Z count (cells x 10^3/μL)	1.56	2.99	2.70	1.68	2.56	1.48
Regions Y + Z count (cells x 10^3/μL)	8.8	14.6	19.8	19.1	13.4	10.5
Timed FCM Z count (cells x 10^3/μL)	5.55	5.75	8.53	7.24	3.34	5.57
Timed FCM Y + Z count (cells x 10^3/μL)	21	23.1	36.4	33.3	18.5	22.2
Manual leukocyte count (cells x 10^3/μL)	6.5	9.4	16.5	13.2	7.1	15.1
Manual leukocyte and thrombocyte count (cells x 10^3/μL)	22.1	62.6	85.2	60.5	29.3	77.1

Note: FCM-generated counts were obtained by multiplying the relative amounts of cells stained with TO in a given region (cells per region/total analyzed) by the total cell count, as obtained by a 1:200 dilution of whole blood in a Neubauer-ruled hemacytometer. Timed counts were obtained by enumerating the number of events in a region per unit time with a flow rate of 60 μL/minute.

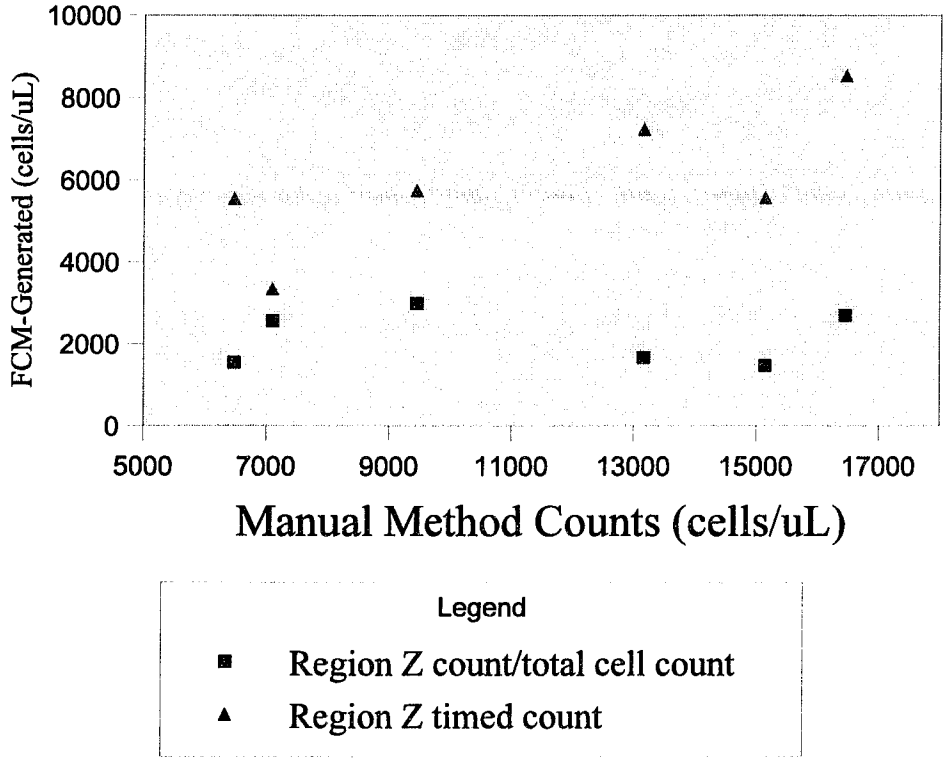

Figure 3.16. Scattergram comparison of leukocyte counts: manual methods vs. FCM-generated (Experiment II) using TO. FCM-generated counts were obtained by multiplying the relative amounts of cells in region Z (cells per region/total analyzed) by the total cell count, as obtained by a 1:200 dilution of whole blood in a Neubauer-ruled hemacytometer. Timed counts were obtained by enumerating the number of events in a region per unit time with a flow rate of 60 μL/minute.

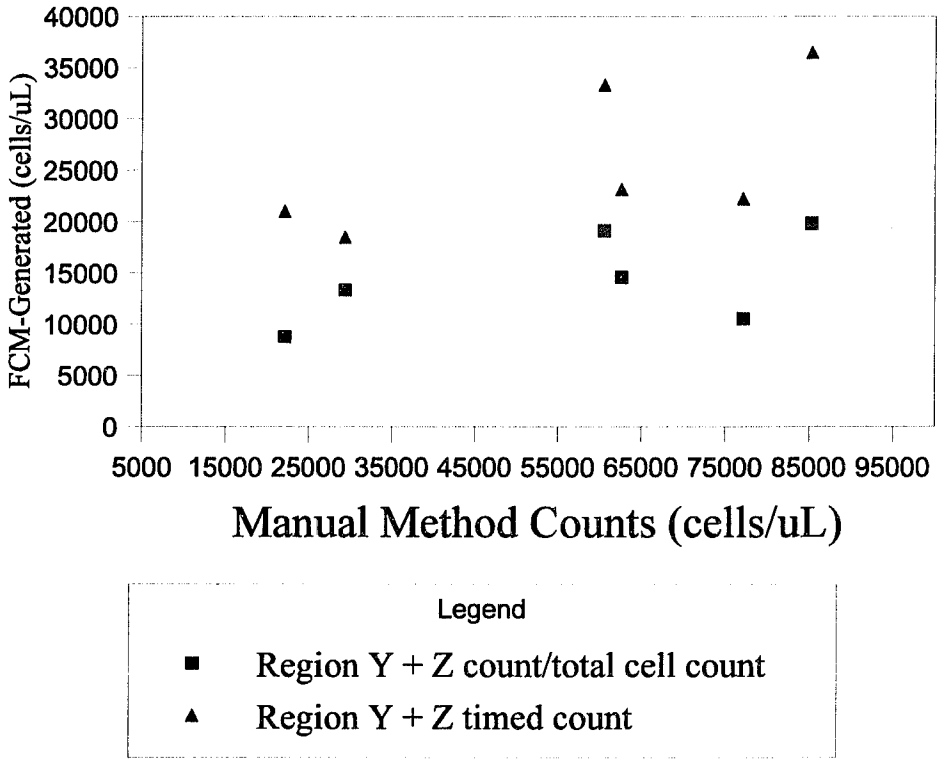

Figure 3.17. Scattergram comparison of combined leukocyte and thrombocyte counts: manual methods vs. FCM-generated (Experiment II) using TO. FCM-generated counts were obtained by multiplying the relative amounts of cells in regions Y+Z (cells per region/total analyzed) by the total cell count, as obtained by a 1:200 dilution of whole blood in a Neubauer-ruled hemacytometer. Timed counts were obtained by enumerating the number of events in a region per unit time with a flow rate of 60 μL/minute.

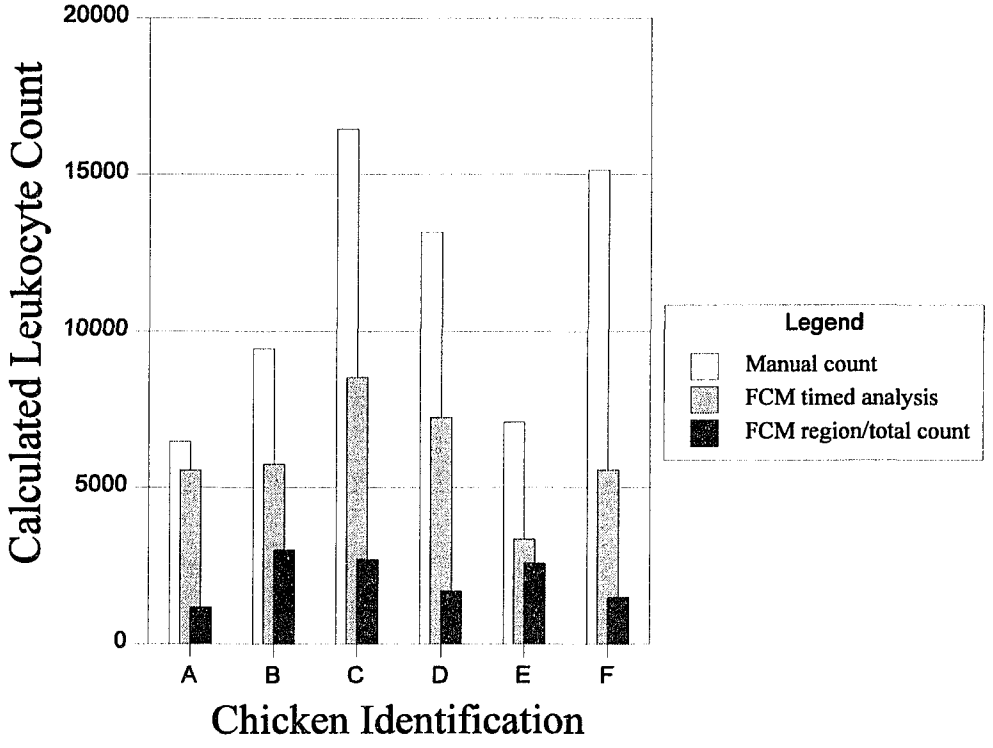

Figure 3.18. Bar graph comparison of manual and FMC-generated total leukocyte counts (Experiment II) using TO. FCM-generated counts were obtained by multiplying the relative amounts of cells in region Z (cells per region/total analyzed) by the total cell count, as obtained by a 1:200 dilution of whole blood in a Neubauer-ruled hemacytometer. Timed counts were obtained by enumerating the number of events in a region per unit time with a flow rate of 60 μL/minute.

either the relative percentage of cells in region Z ($r^2 = 0.006$, slope = -0.479) or the timed analysis ($r^2 = 0.550$, slope = 1.78). When thrombocytes were included in the total count, a linear relationship was still not evident, either with the relative percentage of cells in regions Y and Z ($r^2 = 0.314$, slope = 3.20) or the timed analysis ($r^2 = 0.433$, slope = 2.30). The FCM methods consistently underestimated the total leukocyte count when compared with the manual method (Figure 3.18).

Discussion

Examination of SSC vs. FL-2 dot plots in samples stained with TO resulted in four distinct populations designated Q, X, Y, and Z. The population defined by the region Q most likely represented debris. Data points within this region were found in the low FSC and variably in the SSC areas. This was evident in whole, erythrocyte-, and leukocyte-enriched samples. These areas are usually omitted in FCM examinations by setting using analysis threshold using FSC to exclude the lower channels (72). Cellular debris contains little, if any, nucleic acid to absorb TO, and it is unlikely that a certain blood population would preferentially exclude the dye.

Region X was defined to encompass the vast majority of cells in the whole blood and erythrocyte-enriched samples. Cells delineated by this region were therefore presumably erythrocytes. These cells display a wide variety of FSC and SSC, a property previously demonstrated in avian erythrocytes (47). This population also occurred in leukocyte-enriched samples; however, microscopic examination of cytocentrifuge preparations verified that these samples contained erythrocytes in addition to leukocytes and thrombocytes. The relatively homogeneous and lower

intensity of TO staining of this population conforms with erythrocytes that possess less RNA and more compact DNA than thrombocytes and leukocytes.

Identification of the cells falling within the Y and Z regions was more troublesome. The occurrence of cells in these regions to a much greater extent in leukocyte-enriched samples suggested that they were thrombocytes and leukocytes (Figure 3.3). Low numbers of these events also occurred in the whole blood and erythrocyte-enriched samples. Microscopic examination of the cytocentrifuge preparations also revealed leukocytes in these samples.

Inspection of the colored analysis data also supported this reasoning. Region Y contained a well-delineated population of highly staining cells which were also relatively small (moderately low FSC) and agranular (moderately low SSC). These cells did not fall into the debris (very low FSC/SSC) areas except in hypotonically lysed samples (0.2% NaCl). These characteristics and their relative high concentration suggested that these cells were likely thrombocytes. Mononuclear leukocytes share many morphological characteristics with thrombocytes such as size and density (106), and may also have been located within this region. Cells in the Z region were relatively heterogeneous when examined by FSC and SSC. In fact, cells within this region that stained with the greatest TO intensity were larger (high FSC) and more granular (SSC) than all other cells, which fits with the expectation of monocytes and granulocytes, respectively.

There was some evidence to suggest that region Y and Z may have also contained free nuclei or damaged erythrocytes. Events in these populations were

increased in hypotonic solutions and decreased as the diluent approached 0.9% NaCl (Figures 3.5-3.8). In these samples, the majority of the events in region Y were located in the very low FSC/SSC area. Free nuclei, because of their size and density, scatter relatively little forward and side angle light when compared to intact cells. Additionally, the peak fluorescence channel of the primary distribution in erythrocyte-leukocyte-enriched samples displayed a greater fluorescence intensity in 0.2% samples than 0.9% samples (Figure 3.9; Table 3.2). This indicated that damaged cells, including erythrocytes, retained more TO than intact cells. The increased numbers of events in leukocyte-enriched samples may have simply reflected higher amounts of cell damage secondary to the additional centrifugation steps.

However, there were other indications that these populations did not represent free nuclei or damaged cells. As previously mentioned, cells delineated by these regions in both leukocyte-enriched and whole blood samples diluted in 0.9% saline exhibited a homogeneous cell size, as measured by FSC, that was higher than most erythrocytes in region X and yet stained with much higher intensity. If these events represented free nuclei or damaged erythrocytes, they would be expected to retain low FSC/SSC regardless of diluent, or be widely distributed in the SSC, respectively (47).

The number of events in region Q remained relatively constant throughout the leukocyte-enriched samples, regardless of the diluent concentrations. If Y and Z regions also contained debris, then the amount within Q should parallel the increase in these regions, which was not observed.

Populations staining with higher TO intensity may have also represented a subpopulation of intact erythrocytes, such as reticulocytes. Indeed, TO was first suggested as a reticulocyte dye (125). Lucas and Jamroz reported that reticulocyte counts in chicken blood may range from 7% to 28% in young chickens, but are rare in healthy adult birds (187). Quantitative difference in RNA and DNA in avian reticulocytes and leukocytes has not been reported. Reticulocyte counts were not performed on the blood samples from these chickens. However, if cells within regions Y or Z represented reticulocytes, then thrombocytes and leukocytes, also containing these nucleic acids, should have been as easy to identify.

These FCM methods did not produce total leukocyte counts comparable to currently accepted manual methods. Several potential problems exist. The presence of free nuclei, cellular debris, and erythrocyte subpopulations may have obscured the identification of the leukocytes and thrombocytes. However, if erythroid cells or debris were included in regions containing thrombocytes and leukocytes, the calculated total leukocyte count would have been artificially inflated. This was not evident in these investigations; both types of FCM-generated leukocyte counts were lower than the manual counts (Figure 3.18).

The more likely explanation remains that not all leukocytes stained with demonstrably greater intensity than erythrocytes, and were therefore concealed in the erythrocyte peak. This could be investigated using cell sorting, which allows the physical separation of cells according to population characteristics such as high or low fluorescence intensity during FCM analysis and subsequent microscopic evaluation.

Unfortunately, the avidity of TO for the plastic components of FCM instruments precluded its use in instruments available for these studies. A separate set of fluidics or a dedicated instrument would allow sorting based on TO fluorescence to confirm the identity of populations of interest. In addition, a loss of thrombocytes in the supernatant fluid during centrifugation may has also accounted for decreased numbers of non-erythrocytes in the FCM methods (188).

Another source of error in these investigations was the relatively insensitive methods used for comparison. Other authors have noted the limitations in using manual methods in calibrating automated hematology instruments (67,72). This raised the possibility that the cell counts generated by FCM analysis may not have correlated well with the manual methods simply because of the inaccuracy inherent in the latter. However, this would more likely have been reflected in a closer correlation of the two methods or at least a consistently positive slope. Again, visual inspection of the cells falling within the regions of interest by cell sorting would substantiate this potential problem.

Although these investigations provided evidence to suggest that avian thrombocytes and/or leukocytes preferentially stain with thiazole orange, it was apparent that the methods utilized do not provide a means by which to calculate the total leukocyte count. Additional trials, utilizing cell sorting, may increase the potential use of this dye and method in determining this valuable index in non-mammalian species.

Chapter 4: Differentiation of Chicken Erythrocytes and Leukocytes with Antibodies Directed Against Cytoskeletal Proteins

Introduction

The complete blood count (CBC), including a total leukocyte count, is of paramount diagnostic value during clinical evaluation of diseased animals. Techniques of determining this vital index in non-mammalian species are manual, and include indirect, direct, and semi-indirect methods. Indirect methods simply estimate the number of leukocytes relative to erythrocytes on a blood smear. Direct methods incorporate specific stains to facilitate leukocyte identification in a hemacytometer. The semi-indirect method, popular in diagnostic laboratories (77), utilizes the stain phloxine to identify heterophils and eosinophils in a hemacytometer. The total leukocyte count is then derived by relating the number of positively-stained cells to the heterophil and eosinophil number in the differential count (96).

Because of the small numbers of cells analyzed, these methods are wrought with statistical error (68). In addition, the error associated with the use of hemacytometers in enumerating blood cells can be as high as 30% (105). Slight irregularities in smear preparation can also significantly alter the validity of these results (96,102).

Automated hematology analyzers have been utilized in mammalian diagnostic medicine for decades, resulting in dramatic improvements in both precision and accuracy, as well as cost savings (19,68,94). However, the presence of nuclei in non-

mammalian erythrocytes and thrombocytes precludes the use of currently manufactured instruments (102,189).

Modern hematology analyzers are based on the principles of flow cytometry (FCM). Basically, a flow cytometer consists of a system of fluidics that isolates a suspension of cells into a single stream. Information about each individual cell is collected by detection of light scatter and fluorescence by the interaction with lasers or other strong light sources (4). This information has been correlated with cellular characteristics such as size, density, and antigen content (7). The speed and sensitivity offered of flow cytometric systems has resulted in widespread use and continued expansion of applications (5).

Fluorochrome-labeled antibodies have been used in considerable applications in FCM systems (37). Cells containing antigens of interest can be tagged by labeled antibodies and then identified as high fluorescence intensity events (5). Monoclonal antibody production has escalated the employment of FCM to distinguish cells in suspension bearing specific antigens (4,5). The use of FCM has even been advocated in screening monoclonal antibodies (43). The dramatic impact on immunological research by FCM has probably resulted in more instrument purchases for this type of analysis than any other (190).

Many assays using flow cytometric immunofluorescence involve blood cells; immunomarkers have been created for identification of developing and mature erythroid, myeloid, and lymphoid cells (191). Immunophenotyping has permitted the classification and enumeration of subsets of T, B, and NK cells based on derivation,

development, and function (5,10,192,193). This has resulted in increased knowledge of leukemia and deficiency diseases and their diagnosis (5,194-196).

Mature avian erythrocytes contain three primary cytoskeletal proteins: spectrin, tubulin, and vimentin (128). Spectrin has been described as the major membrane skeletal protein in many cells, including erythrocytes (128-130). Composed of α- and β-subunits, it provides conformational stability in conjunction with many integral cellular proteins (131-134). The different subunits give this protein its variable immunological cross-reactivity with spectrin originating from other tissues or species (131,133,135-137). It is the β-subunit that imparts tissue-specificity, with three different isoforms identified in erythrocytes, brain, or intestinal brush border cells (131,136). Cross reactivity is more likely with polyclonal antibodies that may recognize either of the two subunits, such as that seen in avian tissues labeled with anti-spectrin of mammalian origin (133).

Intermediate filaments of vimentin subunits suspend the nucleus in nucleated erythrocytes by attachments to the membrane skeleton and the nuclear membrane (128,139-141). These proteins contribute to the architecture maintaining the biconvex shape of the mature erythrocyte and may also be involved in the nuclear retention in non-mammalian erythrocytes (128,139,142). Considerable homology has been reported between avian erythrocyte and muscle vimentin (143).

The marginal band (MB) is a well-described microtubular structure of nucleated erythrocytes (128,147), thrombocytes (146,148-150), and mammalian erythrocyte precursors, platelets (128,152-155), and mature camelid erythrocytes

(156). This tubulin band encircling the equatorial rim serves to maintain shape and structural integrity (128,147,156,157) by providing tension across the membrane skeleton (158). It is composed primarily of α- and β-subunits (159), the latter of which is immunologically distinct from β-tubulins in other chicken tissues (146,151,160).

Although non-erythroid spectrin has been identified in mammalian monocytes (133), the structural protein differences in chicken leukocytes are not well documented. Descriptions of the ultrastructure of chicken leukocytes by electron microscopy note the absence of the MB in leukocytes (148-150), although microtubules associated with centrioles have been observed (150). However, there are no studies that address the quantitative and immunological differences of cellular structural proteins in avian erythrocytes, thrombocytes, and leukocytes.

These studies investigated the differences in the cytoskeletal content of chicken erythrocytes and leukocytes. The primary objective was to examine the potential for commercially available antibodies to provide a means of differentiating chicken erythrocytes and leukocytes, and thus form a basis for calculating a total leukocyte count. Using a fluorescein-labeled secondary antibody, a fluorescence threshold could be established to exclude erythrocytes, and potentially thrombocytes. The remaining cells could then be enumerated given a cell count per unit time and constant flow rate. Immunocytochemistry and immunofluorescence microscopy were used to screen methods prior to flow cytometric analysis.

Glutaraldehyde-fixed chicken erythrocytes are frequently used for calibrating fluorescence in FCM (57,196,197). These cells demonstrate a relatively constant induced fluorescence when fixed in 2.5% glutaraldehyde. To examine this phenomenon and the potential use of glutaraldehyde-based fixation methods, both separated and whole blood were fixed in glutaraldehyde and examined via fluorescent microscopy.

Materials and Methods

Antibodies

Anti-chicken spectrin antibody was purchased from a commercial source (Sigma Immunochemicals, Sigma Chemical Company, St. Louis, MO). This delipidized, whole antiserum was produced in rabbits against chicken erythrocytes for immunoblotting assays. Reactivity was reported by the manufacturer against both α- and β-subunits. An anti-β-tubulin antibody (clone TUB 2.1, Sigma Immunochemicals, Sigma Chemical Company, St. Louis, MO) was also used. This mouse-origin IgG1 monoclonal antibody utilized rat brain β-tubulin as the immunogen (198), and was reported to cross-react with human, rat, mouse, bovine, and chicken fibroblast β-tubulin. Anti-vimentin antibody (clone V9, BioGenex Laboratories, San Ramon, CA) was a mouse-origin monoclonal immunoglobulin. This antibody was recommended as an internal tissue processing control by the manufacturer. Affinity-purified fluorescein-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA) consisted of horse-origin anti-mouse IgG and goat-origin anti-rabbit IgG.

Sample acquisition

Chicken blood cells

Adult white leghorn hens from the Louisiana State Agricultural Experimental Station, Central Station - Poultry Unit were used as blood donors. Approximately 2 mL of blood was drawn from the cutaneous ulnar vein of two chickens into K_2EDTA coated syringes with 25 gauge needles. The blood was then immediately transferred to K_3EDTA collection tubes (Vacutainer®, Becton Dickinson Vacutainer Systems, Rutherford, NJ).

Antigen-positive control cells

Cultured mouse fibroblasts (NIH/3T3), originating from Swiss mouse embryo cultures (CRL-1658, American Type Culture Collection, Rockville, MD), were used as positive controls for cytoskeletal antigens. These cells were supplied either adhered to 4-chambered glass slides or suspended in media (Dulbecco's modified Eagle's medium with 10% fetal bovine serum) which was adjusted to approximately 1.75×10^6 cells/mL in 0.1 M phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA, Sigma Chemical Company, St. Louis, MO). These preparations were fixed and stained identically to the chicken blood cells.

Cell fixation and staining

Glutaraldehyde fixation

Glutaraldehyde-fixed chicken blood cells were prepared by a modification of the method described by Loike and Silverstein (197). Chicken blood was centrifuged at 200 *g* for 10 minutes. Plasma was decanted and cells were washed twice in PBS,

using centrifugation at 200 *g* for 7 minutes. Twenty volumes of 2.5% glutaraldehyde in PBS were added, mixed gently, and allowed to rock overnight (Aliquot Mixer Model 4651, Ames, Division of Miles Laboratories, Inc., Elkhart, IN). The cells were then washed twice in PBS, and finally resuspended in PBS with 1% NaN_3. A drop of this cell suspension was placed onto glass slides for fluorescent microscopic examination.

Immunocytochemistry

Glass slides coated with 3-aminopropyltriethoxysilane (AAS), as described by Henderson (199), were used to facilitate cell adhesion. Cytocentrifuge preparations (Cytospin®, Shandon Southern, Sewickley, PA) of whole chicken blood (diluted 1:3 in PBS) were fixed by immediate immersion in cold (4° C) methanol for 10 minutes. Slides were then allowed to air dry and stored at -10°C until use.

Chicken blood cells and NIH/3T3 cells were stained using the avidin-biotin complex in a modification of the procedures described by Hsu, *et al.* (200). Endogenous peroxidase activity was blocked by covering the affixed cells with approximately 500 µL H_2O_2 (0.3% in methanol) for 30 minutes at room temperature (RT). Approximately 500 µL primary antibody (anti-vimentin, 1:50; anti-tubulin, 1:50; anti-spectrin, 1:100 in PBS-BSA) was placed onto the slide surface and allowed to incubate 30-60 minutes at RT. Slides were then washed with PBS. Normal horse serum (1:50 in PBS, Vector Laboratories, Burlingame, CA) was then used in some preparations to block non-specific secondary antibody staining. Five hundred µL biotinylated secondary antibody (anti-mouse, 1:200; anti-rabbit, 1:50 in PBS) was

allowed to incubate on the cells for 30 minutes. Streptavidin-biotin complex (Vector Laboratories, Burlingame, CA) labeled with horseradish peroxidase was then allowed to incubate on the slides for 30-45 minutes at RT. Diaminobenzidine (DAB) or 3-amino-9-ethyl carbazole (AEC) (Sigma Chemical Co., St. Louis, MO) were used as chromagens and were allowed to incubate until a color change was visualized or for 15 minutes. These markers produce brown and red deposits at the enzyme-antibody sites, respectively. Slides were then counterstained with either Mayer's hematoxylin or Giemsa. Coverslips for slides with AEC were mounted with an aqueous medium (Immu-mount, Shandon, Pittsburgh, PA), and for DAB with a resinous medium (CoverBond™, Scientific Products, McGaw Park, IL) following dehydration.

Additional chicken blood samples were stained using alkaline phosphatase as the enzyme marker with the procedures described by Cordell, *et al.* (201). Slides were treated identically, excluding the endogenous peroxidase blocking step. All samples were treated with normal horse serum to block non-specific secondary antibody staining. Steptavidin-biotin complex labeled with alkaline phosphatase was incubated 30-45 minutes at RT. The slides were then incubated 5-15 minutes in naphthol AS-BI phosphate and New Fuchsin containing levamisole, which deposits a red reaction product at the enzyme site. Slides were then counterstained with either Mayer's hematoxylin or Giemsa; coverslips were mounted with a resinous medium (CoverBond™, Scientific Products, McGaw Park, IL) following dehydration.

Negative controls consisted of NIH/3T3 cells stained as above with the omission of the primary antibody step. Chicken blood cells were also stained without primary antibody to demonstrate any non-specific staining.

Immunofluorescence

Twenty μL of whole chicken blood from two birds were diluted in 10 mL PBS. These cells were washed of plasma by centrifugation at 200 *g* for 8 minutes. Samples of NIH/3T3 cells suspended in culture medium were similarly centrifuged and washed in PBS. Both of these samples were then fixed and stained by a modification of the procedures described by Jacobberger *et al.* (202). The cells were resuspended in 100 μL PBS-BSA and mixed gently (Vortex-Genie 2, Model G-560, Scientific Industries, Inc., Bohemia, NY) to suspend the pellet, fixed in 900 μL methanol, and incubated at 4° C for 10 minutes. The fixed cells were then washed in PBS-BSA.

Normal goat or horse serum was employed to block non-specific secondary antibody binding, in samples using rabbit or mouse origin primary antibodies, respectively, by adding 1.0 mL (1:50 in PBS) and incubating 30 minutes at RT in half of the samples. After a PBS-BSA wash, all samples except the negative control were suspended in 1.0 mL primary antibody solution (anti-β-tubulin, 1:50; anti-spectrin, 1:50) and incubated 30 minutes at RT. After washing, the cells were suspended in 1.0 mL of the FITC-conjugated secondary antibody solution (anti-mouse IgG, 1:100; anti-rabbit IgG, 1:100) and incubated 30 minutes RT on a shaker (Red Rotor, Model PR 70, Hoefer Scientific Instruments, San Francisco, CA) in the dark. After a final rinse, the cells were suspended in 50 μg/mL solution of propidium iodide (PI) to

facilitate identification of nuclei for fluorescent microscopy. One drop of each of the samples was used to make a cytocentrifuge preparation and examined via fluorescent microscopy. The remaining sample was placed in polystyrene tubes (Falcon® 2054, Becton-Dickinson Labware, Becton, Dickinson, and Company, Lincoln Park, NJ) for FCM analysis.

As another positive control procedure, NIH/3T3 cells adhered to glass slides were fixed in methanol and stained. In each of the four chambers various staining combinations were used, including with or without blocking with normal goat serum, two sources of secondary antibody, two concentrations of secondary antibody (1:10 and 1:100), and with or without primary antibody (negative control).

In other experiments, air-dried chicken blood smears and NIH/3T3 cells were fixed by additional methods described by Osborn and Weber (162), including acetone (4°C, 10 minutes); formaldehyde (3.7% in PBS, 10 min at RT) with Triton X-100™ (0.2% in PBS, 2 minutes at RT, Union Carbide Chemicals and Plastics Co., Inc., Sigma Chemical Co., St. Louis, MO) following a rinsing step; and methanol with EGTA added (5 mM, -10° C, for 6 minutes). These slides were then stained with approximately 0.1 mL primary (1:100 in PBS) and secondary (1:50 in PBS) antibodies for 45 minutes. Coverslips were mounted with an aqueous medium (Immu-mount, Shandon, Pittsburgh, PA) for fluorescent microscopy.

Analysis of fluorescence

Fluorescent microscopy

Incident light fluorescent microscopy (Ortholux II™, Leitz Wetzlar, E. Leitz, Inc., Rockleigh, NJ) utilized a 200 watt high-pressure mercury vapor lamp as a light source (Model 050222, E. Leitz, Inc., Rockleigh, NJ). This light source was equipped with a multi-wavelength fluorescence illuminator (Pleomopak, E. Leitz, Inc., Rockleigh, NJ). Barrier and exciter filters were inserted in the light path to exclude shorter blue wavelengths to maximize fluorescein emission detection.

Flow cytometry

Flow cytometric analysis of the separated blood samples was performed with a commercial instrument designed primarily for immunological studies (FACScan™, Becton Dickinson Immunocytometry Systems, Becton, Dickinson, and Co., San Jose, CA). This unit used a 15 mW, air-cooled, 488 nm argon ion laser and sterile physiologic saline as sheath fluid. Detection was supplied by photomultiplier tubes as: FL-1 (green fluorescence), FL-2 (orange fluorescence), FL- 3 (red fluorescence), SSC (side-scatter), and FSC (forward scatter). Fluorescence detectors were amplified logarithmically and incorporated a 530/30 nm and 585/42 nm band pass filter for green and orange, and a 670 nm long pass filter for red fluorescence. Data was acquired in list mode to a computer interface (Hewlett-Packard HP 9000 series, model 340, San Diego, CA) and analyzed using supplied software (Lysys™ II, Becton Dickinson Immunocytometry Systems, Becton Dickinson and Company, San Jose, CA).

Results

Glutaraldehyde-fixed cells

Fluorescent and light microscopy of glutaraldehyde-fixed chicken blood cells revealed alterations of erythrocyte morphology. Shapes varied, with most cells distinctly pointed at either one or both poles. Under fluorescent microscopy erythrocytes displayed a significant cytoplasmic fluorescence (Figure 4.1)

Immunocytochemical analysis

Results of the immunocytochemistry experiments are summarized in Table 4.1 and 4.2. Chicken erythrocytes demonstrated no discernable reactivity with the anti-vimentin, spectrin, and β-tubulin. In samples in which the normal serum blocking step was omitted, leukocytes stained positively using cytoskeletal protein antibodies, horseradish peroxidase, and DAB (Figures 4.2, 4.3, and 4.4), including the negative control sample in which no primary antibody was added (Figure 4.5). Mononuclear cells stained with the highest intensity, followed by granulocytes, with thrombocytes staining weakly. However, when normal serum protein was used as a blocking agent, specific reactivity was extremely diminished (Figure 4.6 and 4.7), except for slight staining of granulocytes using the alkaline phosphatase/naphthol AS-BI/New Fuchsin protocol (Figures 4.8 and 4.9). The NIH/3T3 cells stained well with anti-β-tubulin and anti-spectrin antibodies, but not with anti-vimentin, regardless of chromogen or inclusion of blocking steps (Figures 4.10, 4.11, 4.12, and 4.13).

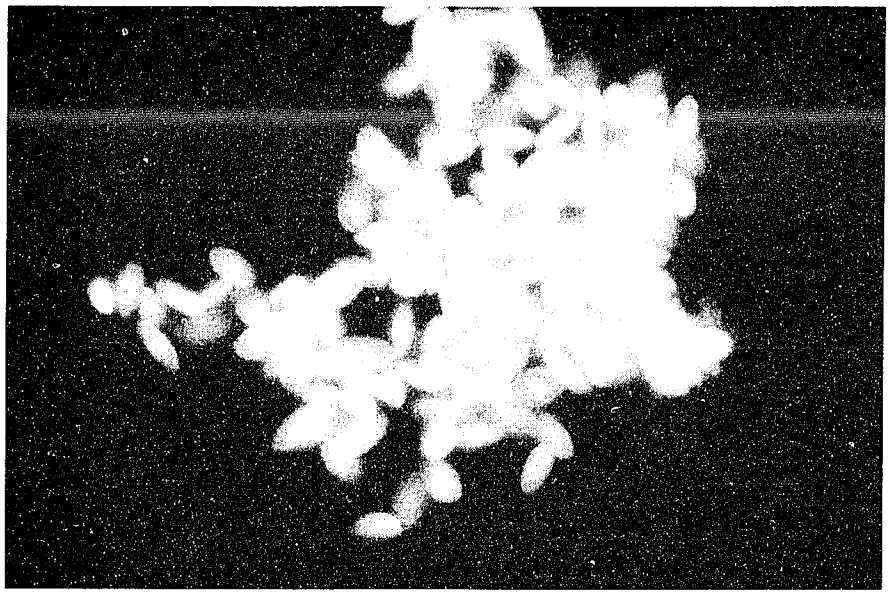

Figure 4.1. Glutaraldehyde-fixed chicken blood cells. Note induced pointed shape and cytoplasmic fluorescence of many erythrocytes.

Table 4.1. Non-specific immunocytochemical staining of chicken blood cells omitting normal serum blocking steps

	NIH-3T3	RBC	Mono	Lymph	Granulo	Thromb
α-vimen	+/-	-	+	+	+/-	+/-
α-spect	+/-	-	+/-	+/-	+/-	-
α-tubul	+	-	+	++	+	+/-
negative	-	-	-	-	-	-

NIH-3T3=cultured mouse fibroblasts; RBC=chicken erythrocytes; Mono=chicken monocytes; Lymph=chicken lymphocytes; Granulo=chicken granulocytes; Thromb=chicken thrombocytes; α-vimen=anti-vimentin 1° Ab; α-spect=anti-spectrin 1° Ab; α-tubul=anti-β-tubulin 1° Ab; negative=negative control (no 1° Ab). - = no staining evident; +/- = slight or variable staining; + = positive staining; ++ = strong positive.

Table 4.2. Immunocytochemical staining of chicken blood cells

	NIH/3T3	RBC	Mono	Lymph	Granulo	Thromb
α-tubul HP-DAB	+	-	+/-	+/-	+/-	-
α-tubul HP-AEC	ND	-	-	-	-	-
α-tubul AP-NF	ND	-	-	-	+/-	-
negative HP-DAB	-	-	-	-	-	-
negative AP-NF	ND	-	-	-	-	-

NIH-3T3=cultured mouse fibroblasts; RBC=chicken erythrocytes; Mono=chicken monocytes; Lymph=chicken lymphocytes; Granulo=chicken granulocytes; Thrombo=chicken thrombocytes; α-tubul=anti-β-tubulin 1° Ab; HP-DAB=horseradish peroxidase conjugated 2° Ab with DAB; HP-AEC=horseradish peroxidase conjugated 2° Ab with AEC; AP-NF=alkaline phosphatase conjugated 2° Ab with New Fuchsin; negative=negative control (no 1° Ab). - = no staining evident; +/- = slight or variable staining; ND = not done.

Figure 4.2. Chicken granulocytes and monocytes stained with anti-β-tubulin, horseradish peroxidase - DAB (non-specifically). Note that no protein blocking step was performed on this sample.

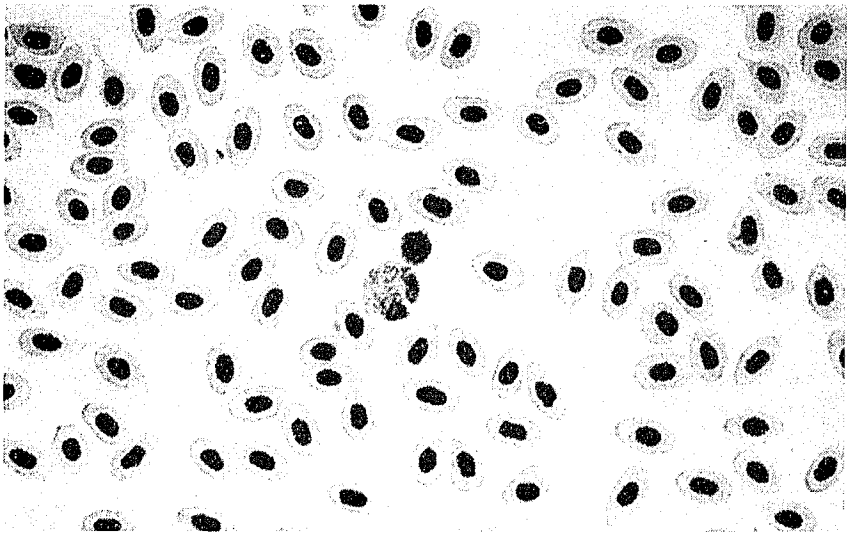

Figure 4.3. Chicken granulocyte and lymphocyte stained with anti-spectrin, horseradish peroxidase - DAB (non-specifically). Note that no protein blocking step was performed on this sample.

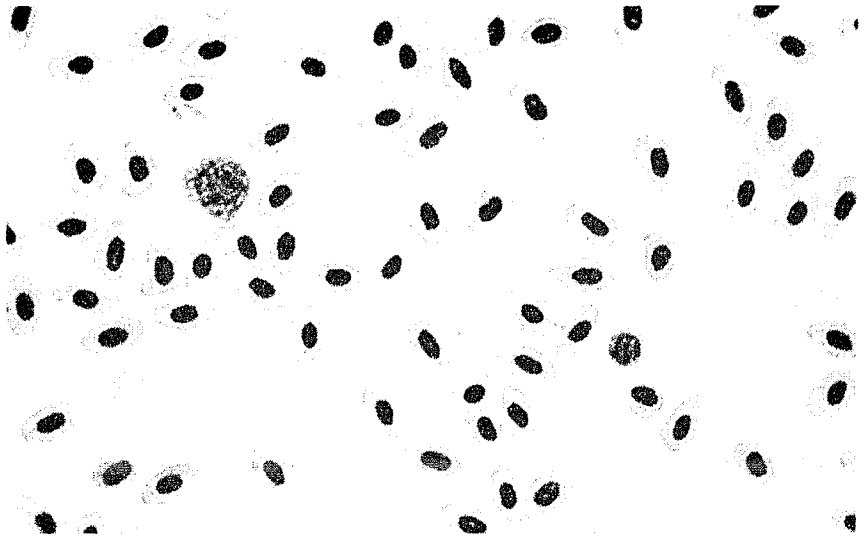

Figure 4.4. Chicken monocyte and lymphocyte stained with anti-vimentin, horseradish peroxidase - DAB (non-specifically). Note that no protein blocking step was performed on this sample.

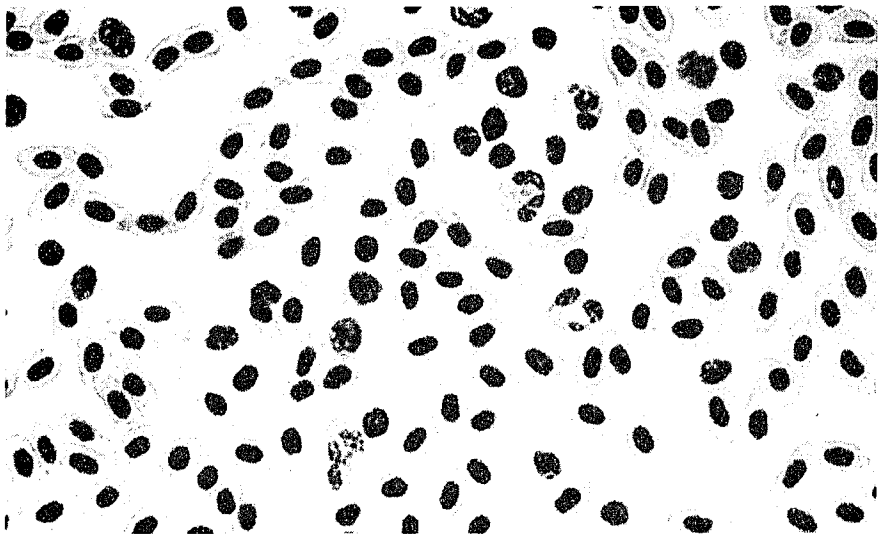

Figure 4.5. Chicken leukocytes stained substituting PBS for primary antibody, horseradish peroxidase - DAB (non-specifically). Note that no protein blocking step was performed on this sample.

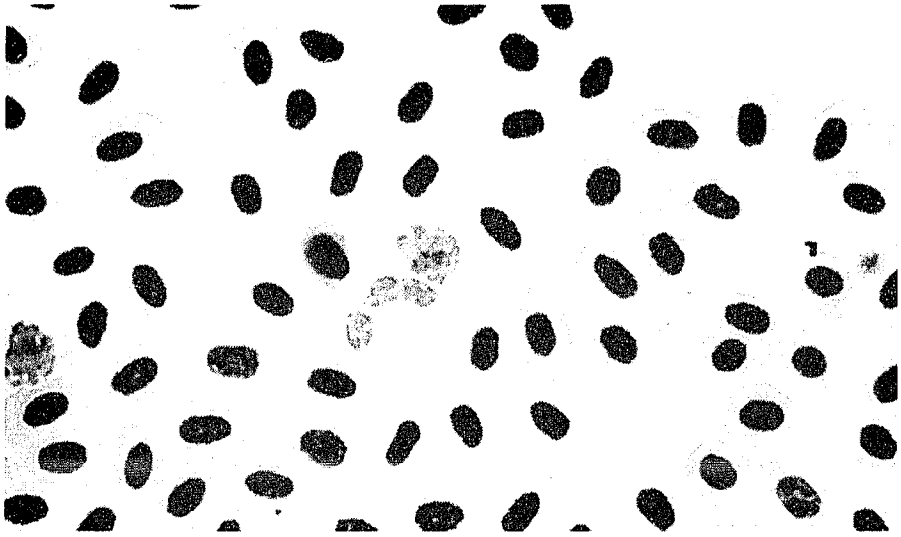

Figure 4.6. Chicken granuloyte and lymphocyte demonstrating lack of staining with anti-β-tubulin, horseradish peroxidase - DAB. Note that a protein blocking step was performed on this sample.

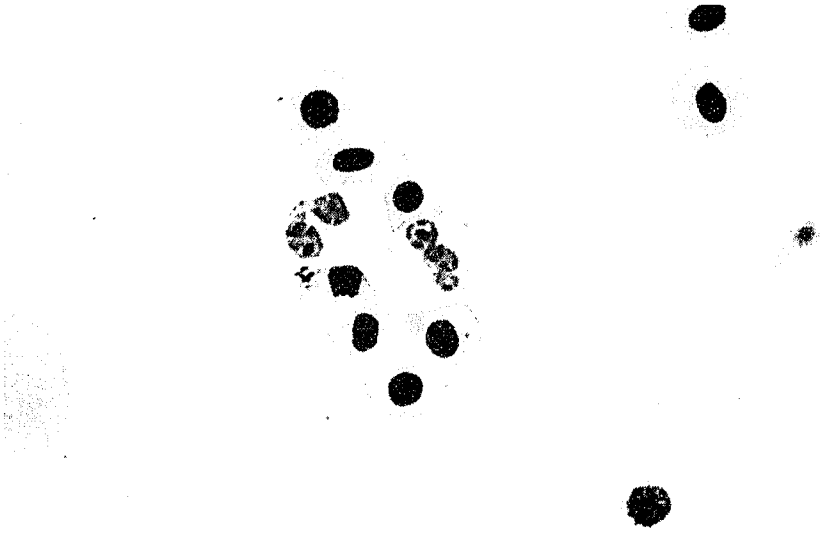

Figure 4.7. Chicken granulocytes demonstrating a lack of staining when PBS was substituted for primary antibody, horseradish peroxidase - DAB. Note that a protein blocking step was performed on this sample.

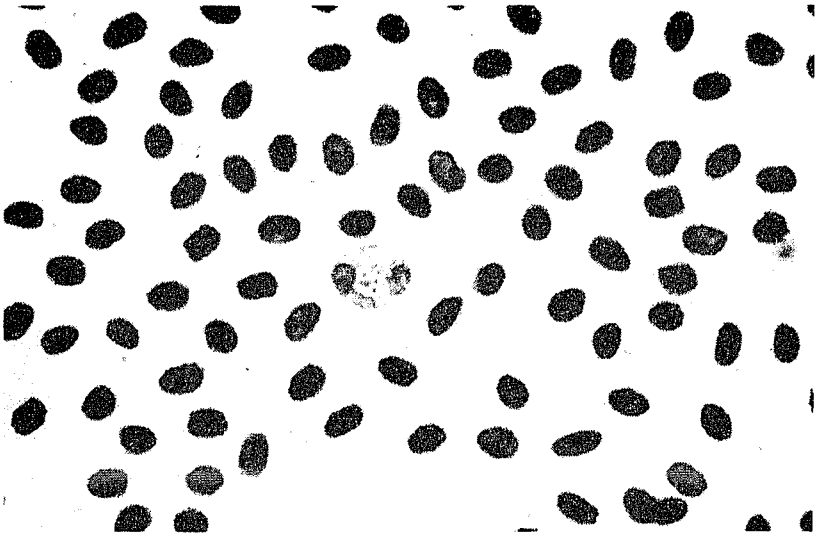

Figure 4.8. Chicken granulocyte demonstrating slight staining with anti-β-tubulin, alkaline phosphatase - New Fuchsin. Note that a protein blocking step was performed on this sample.

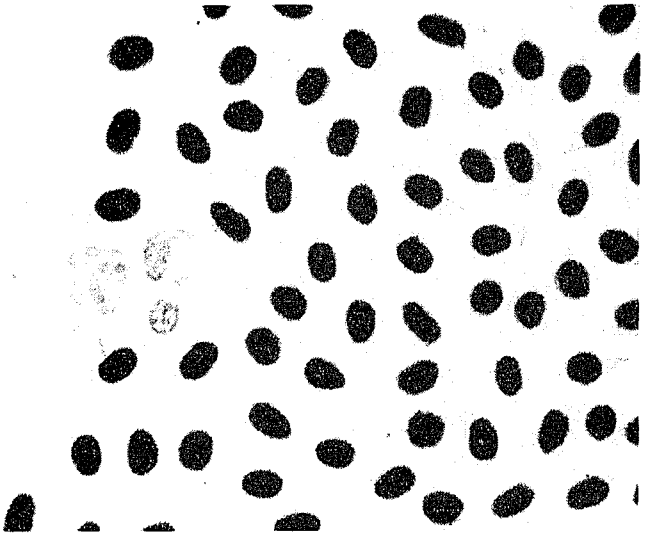

Figure 4.9. Chicken granulocyte and lymphocyte demonstrating lack of staining in negative control samples with no primary antibody, alkaline phosphatase - New Fuchsin. Note that a protein blocking step was performed on this sample.

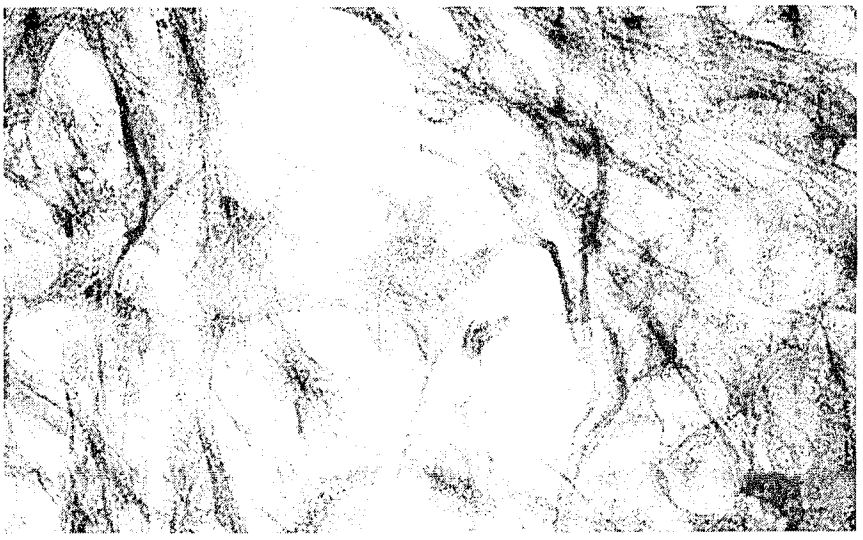

Figure 4.10. NIH/3T3 cultured fibroblast cells stained with anti-β-tubulin, horseradish peroxidase - DAB.

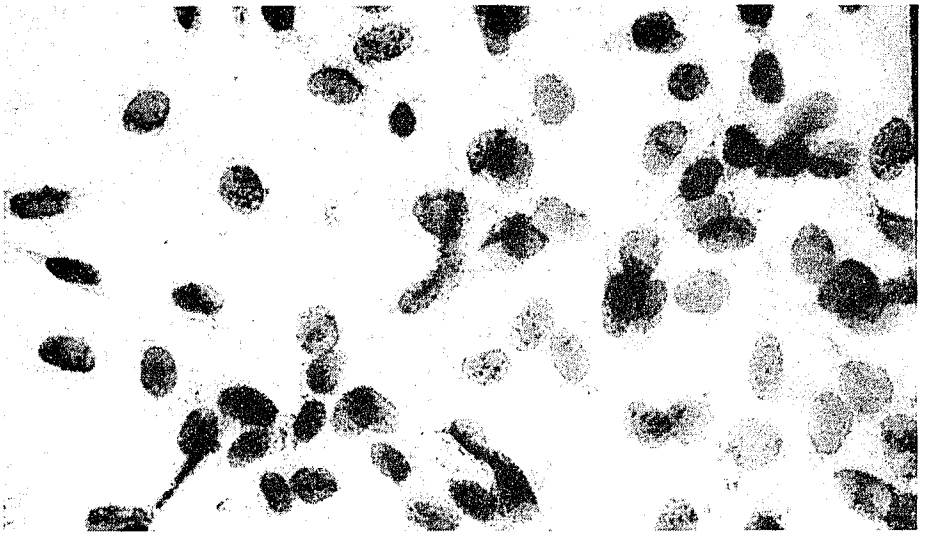

Figure 4.11. NIH/3T3 cultured fibroblast cells stained with anti-spectrin antibody, horseradish peroxidase - DAB.

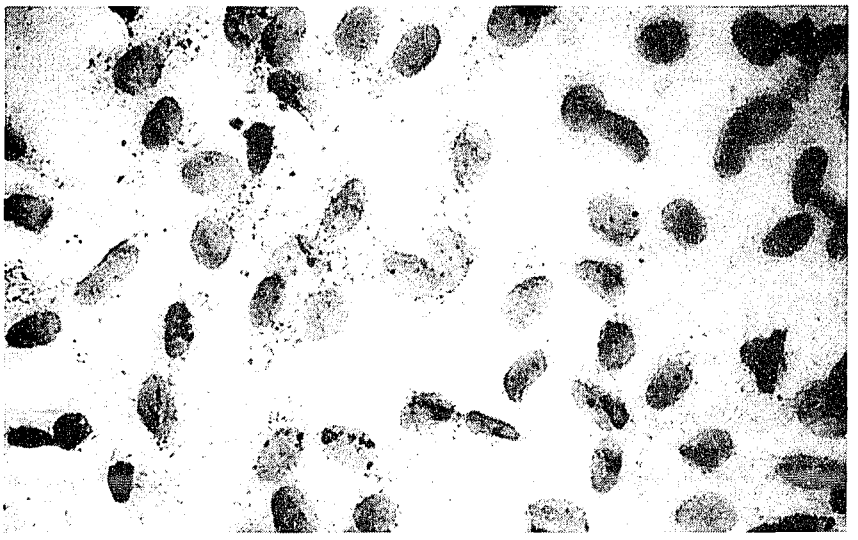

Figure 4.12. NIH/3T3 cultured fibroblasts stained with anti-vimentin, horseradish peroxidase - DAB.

Figure 4.13. NIH/3T3 cultured fibroblasts stained with no primary antibody, horseradish peroxidase - DAB (negative control).

Immunofluorescence

Samples prepared using fixation and permeabilization methods such as methanol/EGTA and formaldehyde/Triton X-100™ with no normal serum blocking step resulted in findings similar to immunocytochemistry experiments. Leukocytes appeared to stain more intensely with both anti-spectrin and anti-β-tubulin antibodies (Figure 4.14). The NIH/3T3 controls also stained well with anti-β-tubulin immunoglobulins (Figure 4.15).

Additional investigations of immunofluorescence using anti-β-tubulin were performed using fluorescent microscopy and flow cytometry, but included the blocking step using normal horse serum. When viewed by fluorescent microscopy, all cells were easily identified by the nuclear staining with propidium iodide, however, no chicken cells exhibited uptake of FITC-labeled antibody (Figure 4.16). The NIH/3T3 fibroblasts, stained both adhered to slides and in suspension, contained a cytoplasmic fibrillar pattern of fluorescence (Figure 4.17).

Samples of chicken blood and NIH/3T3 cells stained with anti-β-tubulin were analyzed by FL-1 fluorescence intensity in the flow cytometer. Suspension of NIH/3T3 cells demonstrated a population of much higher fluorescence intensity when compared to controls with no primary antibody (Figure 4.18). Although staining chicken whole blood increased the FL-1 intensity, no small populations consistent with leukocytes were present in the distributions (Figure 4.19).

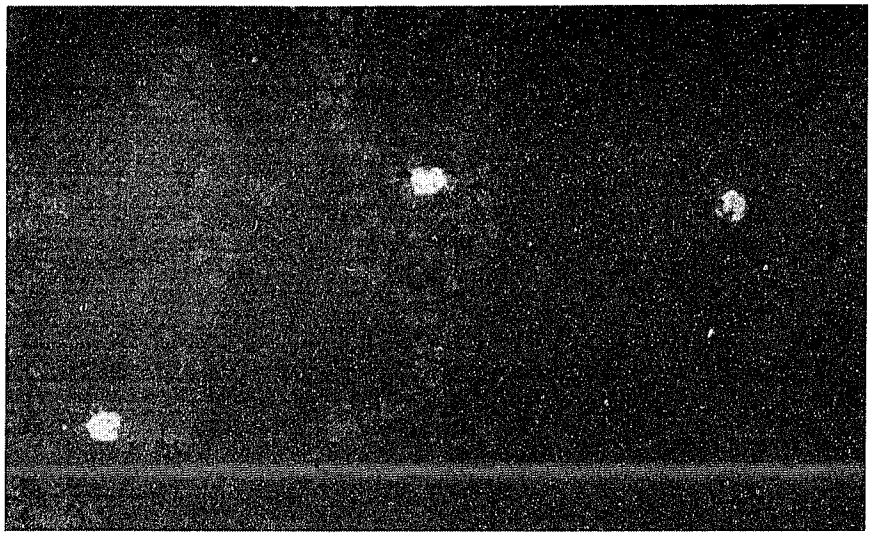

Figure 4.14. Chicken leukocytes stained with anti-β-tubulin and FITC-conjugated secondary antibody (non-specifically). Note that no protein blocking step was performed on this sample.

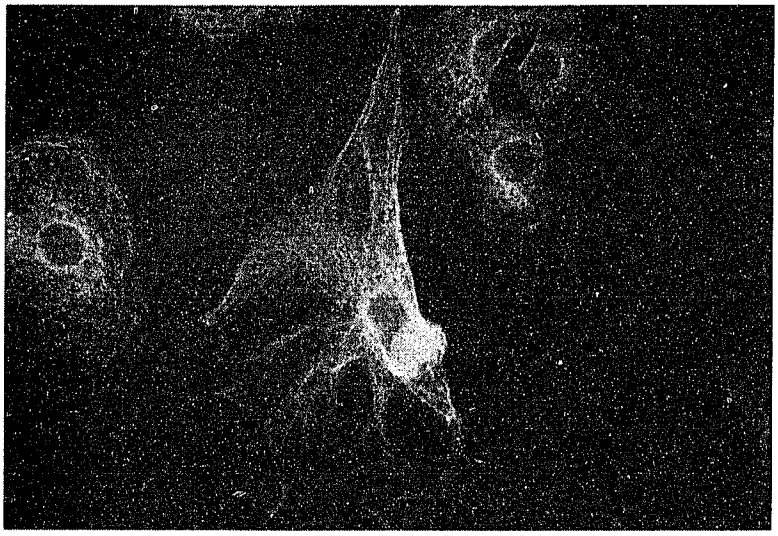

Figure 4.15. NIH/3T3 cultured fibroblasts stained with anti-β-tubulin and FITC-conjugated secondary antibody.

Figure 4.16. Chicken blood cells stained with anti-β-tubulin, FITC-conjugated secondary antibody, and PI using a protein blocking step. Note the leukocyte in the center of the view demonstrating no appreciably higher cytoplasmic fluorescence than the surrounding erythrocytes.

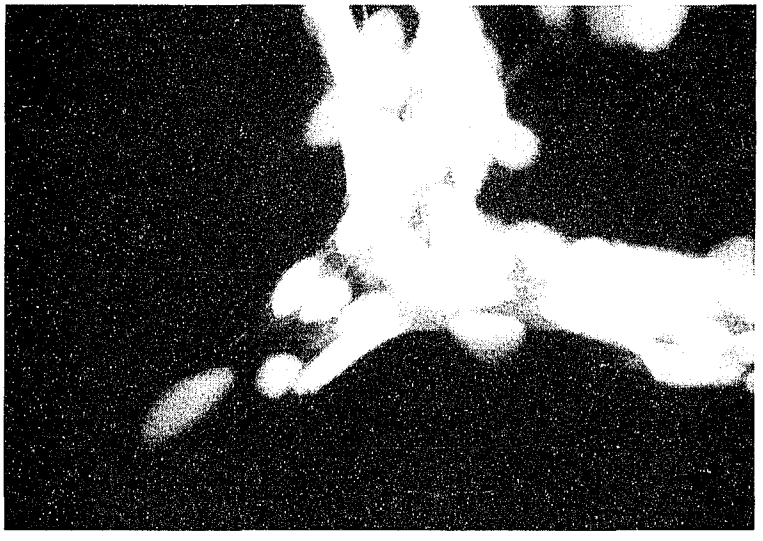

Figure 4.17. NIH/3T3 cultured fibroblasts stained with anti-β-tubulin, FITC-conjugated secondary antibody, and PI. Note the distinctive cytoplasmic fluorescence, especially where cells become confluent.

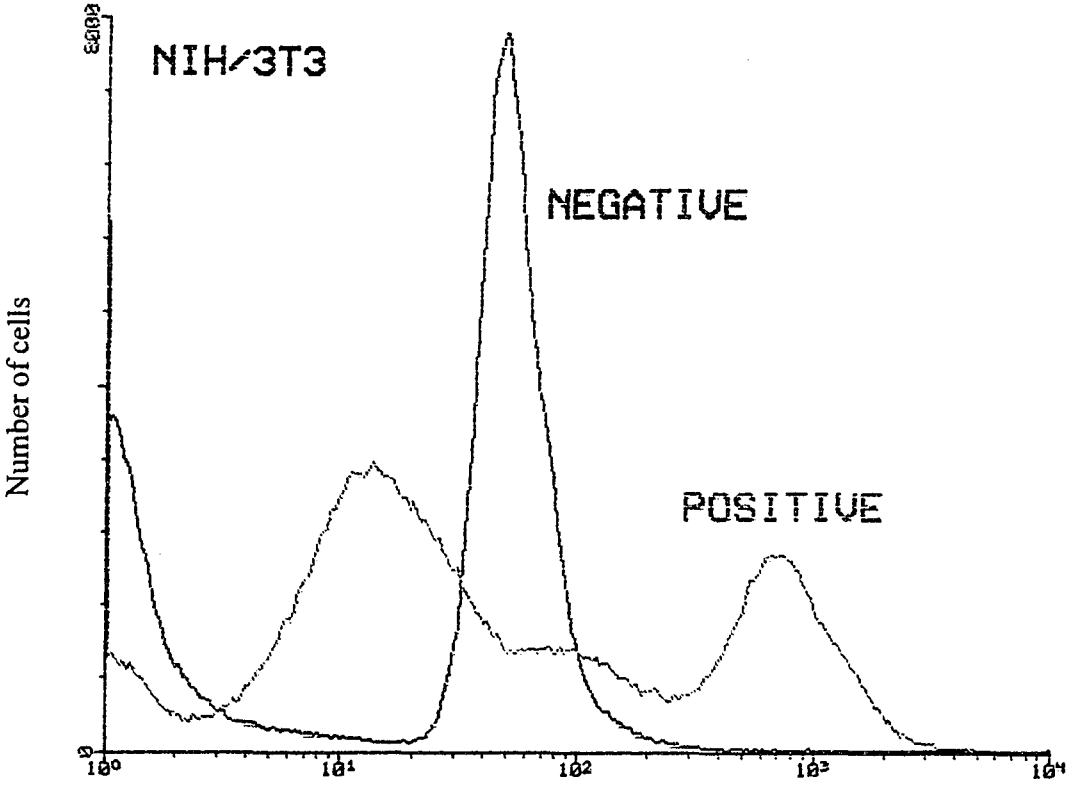

Figure 4.18. FL-1 fluorescence frequency histogram of NIH/3T3 cultured fibroblasts stained with anti-β-tubulin and FITC-conjugated secondary antibody. Note the population of high intensity cells in the positive sample. Negative samples used PBS substituted for primary antibody.

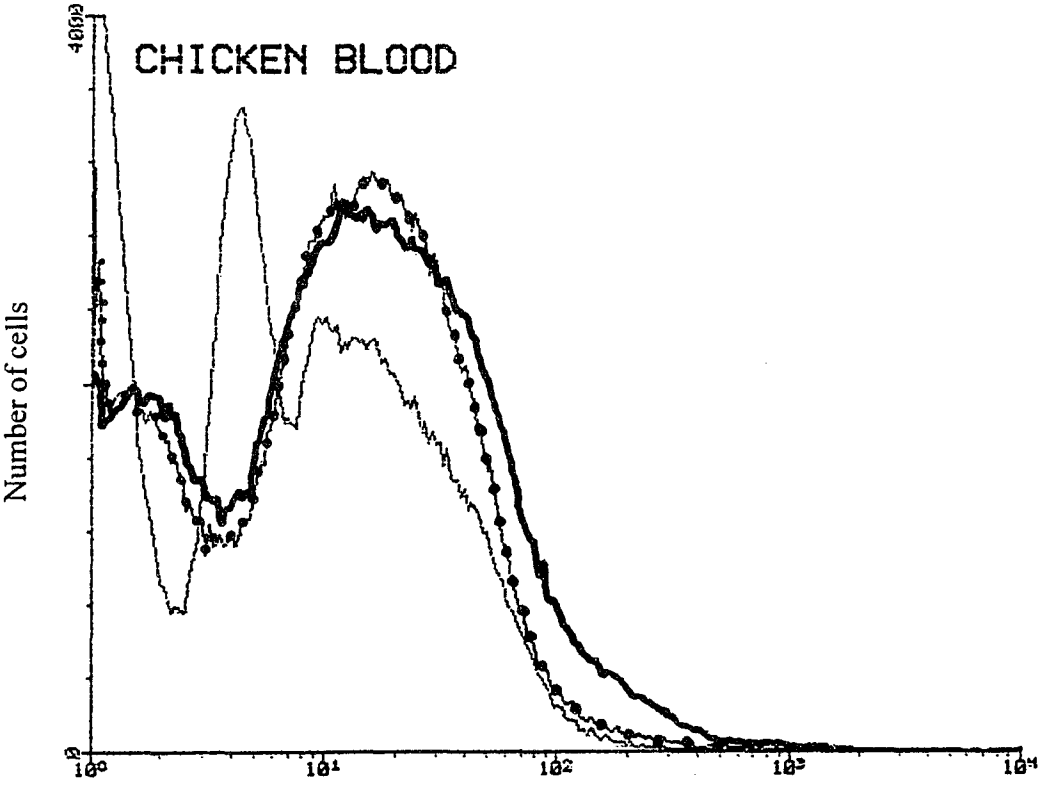

Figure 4.19. FL-1 fluorescence frequency histogram of chicken whole blood stained with anti-β-tubulin and FITC-conjugated secondary antibody. Note the absence of a smaller population of high fluorescence intensity cells consistent with leukocytes or thrombocytes.
Legend: secondary antibody only (--------); primary + secondary / no protein block (▬▬▬); and primary + secondary / with protein blocking step (-•-•-•-)

Discussion

In initial studies, chicken leukocytes appeared to stain with greater intensity with antibodies directed against cytoskeletal proteins. However, blocking with normal serum greatly diminished staining, indicating that the positivity resulted from non-specific staining. The repertoire of antibodies in the normal serum acts to block non-specific staining by saturating reactive sites that may unexpectedly bind either the primary or secondary antibody.

Non-specific decoration of antigens by immune serum has many sources, each of which may have played a role in the initial reactivity in these studies. Many of these are caused by limitations associated with polyclonal secondary antibodies. Several authors have noted the presence of autoantibodies directed against cytoskeletal proteins in rabbit serum (203-205). Similarly, contaminant antigens in the original immunogen used to produce the either the primary or secondary antibodies may result in immunoglobulins to epitopes also present in the test sample (206). This type of antigenic contamination may have resulted in lack of specificity for chicken spectrin; it is possible that leukocyte antigens were also present in the immunogen. Affinity purification is usually employed to remove contaminate antibodies in commercial preparations. However, this purification is only as specific as the original immunogen in the affinity column (206). Additionally, fluorescein-conjugated proteins in the secondary antibody preparations may bind to cellular structures regardless of the presence of primary antibody (162,163).

Fluorescein to protein (F:P) ratios are often calculated by the manufacturer of secondary antibodies. Ratios over 2.0 may result in excess non-specific staining by unconjugated FITC in the preparation which may subsequently bind to cellular protein (161,207). The F:P ratio for the secondary antibodies in these investigations was reported as 4.7 for the anti-rabbit and 5.9 for the anti-mouse immunoglobulins. These were sufficient to provide an additional source for non-specific staining.

Various mechanisms of non-specific staining by leukocytes may also be involved. Receptors for Fc components are found on certain chicken and mammalian T-lymphocytes (148). Receptors for both Fc and IgG are found on mammalian monocytes and eosinophils (208,209), which may necessitate the use of affinity purified $F(ab')_2$ fragments (9,161). Additionally, the fluorescein moiety of the secondary immunoglobulin is highly electronegative; electrostatic binding to positively-charged intracellular structures, such as acidophilic cytoplasm and eosinophilic granules, may also occur (210,211). Suggested methods of blocking this endogenous fluorescence have included prior treatment with diaminobenzidine and hydrogen peroxidase (212), which results in hindrance of immunoglobulin binding to eosinophil sites (213), and pretreatment with Lendrum's phenol chromotrope 2-R stain (214). This latter blocking step frequently results in antigenic alterations, however.

It is possible that some of the positivity noted in chicken blood samples not blocked with normal serum was caused by these types of non-specific staining in mononuclear cells, eosinophils, and heterophils. For the purposes of this study, non-specific staining with secondary antibodies would have been as useful as specific

staining, as long as the cells of interest, i.e. leukocytes, could be recognized and enumerated by the flow cytometer. However, this non-specific staining was not apparent on FCM analysis, even in samples in which the blocking step was omitted.

Non-specific staining was greater in the surveillance methods of immunocytochemical methods. Although non-specific reactions due to endogenous enzyme activity are common, this enzyme was blocked with excess substrate (H_2O_2) prior to antibody addition. On the other hand, immunocytochemistry has the potential to be a more sensitive assay than immunofluorescence due to the greater number of fluorochromes present in the enzyme complex in comparison to the number of fluorescein molecules conjugated to the secondary antibody (161). It is therefore possible that the mild reaction seen in leukocytes stained with anti-β-tubulin and horseradish-peroxidase-DAB in blocked samples represented the presence of the antigen in small amounts. Another potential complicating factor is non-specific binding of the avidin-biotin complex to cellular avidin or biotin receptors which may occur (9,161).

Chicken erythrocyte cytoskeletal proteins failed to react with any immunoglobulin. Although the anti-chicken spectrin was reported by the manufacturer to react with erythrocyte spectrin, these studies failed to demonstrate any significant reactivity with this protein. This antibody was recommended for use in immunoblotting assays; how this immunoglobulin reacts *in situ* was not stated. Possible causes of the absence of staining include insufficient membrane

permeabilization, inappropriate fixation, limited detections of mild fluorescence, and lack of cross-reactivity.

Permeabilization of the cytoplasmic membrane is required to allow access of reagents to intracytoplasmic structures (137,162). Fixatives that extract insufficient plasma membrane from erythrocytes may also permit retained hemoglobin to obscure microtubules (147). It is possible that the erythrocyte membrane was inadequately permeabilized, although the fixatives used were previously described for labeling these structures in chickens (137,151,160,162), and functioned well for the NIH/3T3 cells.

Glutaraldehyde has been described as a favorable method of fixation for tubulin-containing cells (162), however, this method induced fluorescence in chicken erythrocytes, potentially interfering with FITC-produced fluorescence. The pointed shape resulting from glutaraldehyde fixation also suggested MB alterations that could modify antigenicity, similar to that described using other aldehyde fixatives (157,162). In other studies, nucleated erythrocytes were lysed by chemical or physical methods such as sonication (140,146,147,157,158), which would likely render the leukocytes unidentifiable. Microtubule stabilization has been suggested, using such chemicals as taxol (145). Again, the NIH/3T3 fibroblasts stained well without such measures.

Methanol has been described as a fixative of choice for immunologically labeling cytoskeletal proteins and was therefore utilized in most of these studies (162). Alcohol fixatives have been used successfully in immunofluorescence studies of several intracellular structures in chicken blood cells (126). Chelation of calcium ions

is often necessary when using immunofluorescence to label tubulin (162), hence EDTA or EGTA was included. Fixation by methanol or ethanol often causes cell aggregation (215). Small amounts of clumping were evident in many samples in this study. Disruption of clumping was performed, often with some difficulty, by agitation; this would represent a serious limitation for use in an automated instrument.

Detection of immunofluorescence is often hindered by interference with the autofluorescence of flavin nucleotides (37). More than 10,000 molecules of FITC-conjugated antibody must be present in an autofluorescent cell, even if signal detection sensitivity is as low as 3000 (162,216). The sensitivity and use of non-stained controls in these studies should have uncovered this potential source of error. Other factors may impede detection of weak signals, such as modification of the antigenic site by manipulation or concealment of the epitope by morphological alterations of the cells (162).

The most lucid sources of failure to detect cytoskeletal proteins in the chicken blood cells were the specific antibodies utilized. The primary antibodies used for these investigations may have simply lacked the cross-reactivity necessary to identify analogous proteins in chicken erythrocytes (162). Weak reactions have been noted when using antibodies to chicken vimentin in human cells, indicating poor cross-reactivity (139). Although a wide spectrum of cross-reactivity among species has been noted for tubulin (217-219), other reports indicate a species specificity (220). The anti-β-tubulin antibody utilized was reported to cross-react with chicken

fibroblasts; however, β-tubulin antigens in these tissues may be antigenically distinct from those in erythrocytes.

The MB can be identified in chicken erythrocytes using polyclonal antibodies directed at brain tubulins (221), however, Murphy, *et al.* indicated that it is more difficult to label using antibodies not specifically aimed at the β-subunit found in erythrocytes and thrombocytes (151). In their report, centromeric tubulins of leukocytes as well as erythrocyte MB tubulin labeled well with general tubulin antibody, which consisted of a rabbit-origin polyclonal antibody against both α and β subunits of tubulin vinblastine crystals from sea urchin eggs purified using chicken brain tubulin. A low-affinity binding of the anti-β-tubulin antibody to chicken leukocyte tubulin may have accounted for some of the positivity reported here. Centrioles and basal bodies have also been strongly labeled by non-immune rabbit serum such as secondary antibody preparations (204).

These investigations indicated that the use of these commercially available immunomarkers was not sufficient to distinguish chicken erythrocytes and leukocytes. The inability of these antibodies to react with chicken blood cells identifies the need for other antibodies with either increased cross-reactivity or specificity against chicken erythrocytes and/or leukocytes.

Immunolabeling methods for cell surface antigens is considerably less tedious than for intracellular structures, however, little work has been performed to investigate these structures in chicken blood cells. Several proteins and glycoproteins have been

described on the surface of chicken erythrocytes (222,223), including the A blood group antigens (224), and others related to the MHC antigens (225).

These A antigens were not found on lymphocytes using purified monoclonal antibodies (226). However, several specific epitopes have been discovered in leukocytes. Using cross-reactivity of antibodies to thymic and bursal cell surface antigens, relationships between chicken lymphocytes and thrombocytes have been suggested (227). An antigen termed MB1 has been labeled in quail hematopoietic cells, leukocytes, and endothelial cells (228). Several CTL antigens found on chicken lymphocytes are biochemically and functionally similar to human and mouse lymphoid antigens (229,230), including an antigen analogous to CD45 (231). This pan-leukocyte protein apparently performs phosphotyrosine phosphatase activity similar to the human antigen. Immunomarkers against this epitope would provide an ideal mechanism of identifying chicken leukocytes.

A major limitation of utilizing this form of identification is the potential of creating a species-specific method. An antibody labeling leukocytes with little or no reactivity to erythrocytes in chickens only would be of little value in a general, non-mammalian hematology instrument. Another potential problem in using immunological methods in routine hematology is the formation of doublets by reactive antibodies causing agglutination of cells (43). These cell clusters would not only be recognized as an additional population of cells staining with twice the intensity (172), but also would artificially lower the cell count. However, the development of a method of calculating this important parameter in non-mammalian species, regardless

of its limitations, would be an important step in encouraging additional research to extend the application to routine diagnostic hematology.

Chapter 5: Summary and Conclusions

These investigations attempted to discover a method of differentiating chicken erythrocytes and leukocytes by standard flow cytometric techniques that could be easily adapted for automated hematology instrumentation. The ability to clearly distinguish these two populations would allow automation of the total leukocyte count, a commonly used parameter in diagnostic medicine not currently available for non-mammalian blood. Using two commercial instruments designed primarily for immunological studies (FACScan™ and FACS 440™, Becton-Dickinson Immunocytometry Systems, San Jose, CA), several extrinsic structural parameters were analyzed, including cytoplasmic and nuclear protein, nucleic acids, and the cytoskeletal proteins spectrin, vimentin, and tubulin.

The first two inquiries utilized fluorescent stains to bind leukocyte cytoplasmic and nuclear contents in preferentially higher quantities. Fluorescein isothiocyanate (FITC) and thiazole orange (TO) have been demonstrated to identify particular cells in heterogeneous samples (52,121-125,185). The binding of these compounds to cellular components fits well into Shapiro's classification of fluorescence mechanisms (60). The fluorescence of FITC establishes contrast with the surrounding medium and negatively-staining cells by washing unbound dye from the system. Conversely, TO exhibits an increase in quantum efficiency when bound to nucleic acids, therefore not requiring a washing step (125). Binding of both fluorochromes is independent of species-specific characteristics, such as cell surface antigens or enzyme activity, and therefore, their expected reactivity in non-mammalian blood cells is high.

Experiments indicated that leukocytes preferentially bind free FITC. This was appreciable by fluorescence microscopy and FCM-generated histograms of leukocyte- and erythrocyte-enriched samples. An increased resolution between enriched samples was achieved using a two-parameter, FL-1 vs. FL-3, dot plot.

Sorting of FITC-stained whole blood revealed significant numbers of leukocytes admixed in the erythrocyte peak. Without the ability to place a minimal fluorescence threshold to ignore the majority of the erythrocytes, a leukocyte peak would be overshadowed, and therefore not reliably assessed.

Both erythrocytes and leukocytes subjected to hypotonic solutions stained with increased FITC intensity. This increased overlap in fluorescence obscured delineation between populations, creating difficulty in setting discriminating thresholds. Although minor amounts would be insignificant for populations of approximately equal concentration, the amount of overlap observed would effectively eliminate identification of the relatively rare leukocytes. Without a clear delineation, any increased fluorescence by damaged erythrocytes during preparatory steps would artificially inflate leukocyte counts.

Examination of forward-scatter, side-scatter, and orange fluorescence of cells falling into three distinct regions indicates that leukocytes and thrombocytes preferentially stain with TO. This was best demonstrated in leukocyte- and erythrocyte-enriched samples. However, manual total leukocyte counts correlated poorly to flow cytometric counts. These FCM-generated leukocyte counts and combined leukocyte/thrombocyte counts were consistently lower than the semi-

indirect manual method, suggesting that some leukocytes failed to stain with discernably increased fluorescence. Similar to FITC studies, the presence of erythrocyte precursors and cellular debris in blood samples may also alter the calculated count using this method by increasing the number of higher fluorescence events.

Immunological studies initially suggested that chicken leukocytes are decorated with antibodies directed against cytoskeletal proteins. This reactivity was subsequently established to be due to non-specific binding of antibody preparations. This non-specific fluorescence was not sufficient to differentiate leukocytes from erythrocytes and thrombocytes by FCM. The cytoskeletal structures were not labeled in erythrocytes, indicating a lack of cross-reactivity between these proteins and the antigens used to produce the immunoglobulins.

Although these investigations verified that standard flow cytometric techniques may be utilized to analyze avian leukocytes, sufficient differentiation of these cells from erythrocytes was not achievable for quantitative purposes. Increased sensitivity of fluorescence detection or specificity of staining methods are required to develop a system by which this important diagnostic evaluation can be automated for non-mammalian hematology.

References

1. Anon. Veterinary health care market for miscellaneous pets. J Amer Vet Med Assoc 1984;184:741-2.

2. Anon. Pet-bird and exotic-pet owners and their use of veterinary services. J Amer Vet Med Assoc 1988;193:1490-1.

3. Porter RE, Wieser MG. Effect of immune-mediated erythrocyte agglutination on analysis of canine blood using a multichannel blood cell counting system. Vet Clin Pathol 1990;19:45-50.

4. Braylan RC. Flow cytometry. Arch Pathol Lab Med 1983;107:1-6.

5. Lovett EJ III, Schnitzer B, Keren DF, Flint A, Hudson JL, McClatchey KD. Application of flow cytometry to diagnostic pathology. Lab Invest 1984;50:115-40.

6. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:78-79.

7. Miller RG, Lalande ME, McCutcheon MJ, Stewart SS, Price GB. Usage of the flow cytometer-cell sorter. J Immun Methods 1981;47:13-24.

8. Lucas AM, Jamroz C, eds. Atlas of Avian Hematology. Agriculture Monograph 25. Washington, D.C.: United States Department of Agriculture, 1961:212-215.

9. Preffer FI. Flow cytometry. In: Colvin RB, Bhan AK, McClusky RT, eds. Diagnostic Immunopathology. New York: Raven Press, 1988:453-73.

10. Coon JS, Landay AL, Weinstein RS. Biology of disease: advances in flow cytometry for diagnostic pathology. Lab Invest 1987;57:453-79.

11. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985.

12. Moldavan A. Photo-electric technique for the counting of microscopical cells. Science 1934;80:188-9.

13. Gucker FT Jr, O'Konski CT, Pickard HB, Pitts JN Jr. A photoelectric counter for colloidal particles. J Amer Chem Soc 1947;69:2422-31.

14. Gucker FT Jr, O'Konski CT. Electronic methods of counting aerosol particles. Chem Rev 1949;44:373-88.

15. Ferry RM, Farr LE Jr, Hartman MG. The preparation and measurement of the concentration of dilute bacterial aerosols. Chem Rev 1949;44:389-417.

16. Caspersson TO. Cell Growth and Cell Function: a Cytochemical Study. New York: W. W. Norton and Company, Inc., 1950.

17. Coulter WH. High speed automatic blood cell counter and cell size analyzer. Proceedings of the National Electronics Conference 1956;12:1034-42.

18. Kubitschek HE. Counting and sizing micro-organisms with the Coulter Counter. In: Ribbons DW, Norris JR, eds. Methods in Microbiology. London: Academic Press, 1969:593-610.

19. Mansberg HP, Saunders AM, Groner W. The Hemalog D white cell differential system. J Histochem Cytochem 1974;22:711-24.

20. Williams LJ. Cell histograms: new trends in data interpretation and cell classification. J Med Technol 198;1:189-97.

21. Mayer K, Chin B, Baisley A. Evaluation of the S-Plus IV. Am J Clin Pathol 1985;83:40-6.

22. Cox CJ, Habermann TM, Payne BA, Klee GG, Pierre RV. Evaluation of the Coulter Counter Model S-Plus IV. Am J Clin Pathol 1985;84:297-306.

23. Shulman G, Yapit MK. Whole-blood platelet counts with an impedance-type particle counter. Am J Clin Pathol 1980;73:104-6.

24. Day HJ, Young E, Helfrich M. An evaluation of a whole-blood platelet counter. Am J Clin Pathol 1980;73:588-93.

25. Cairns JW, Healy MJR, Stafford DM, Vitek P, Waters DAW. Evaluation of the Hemalog D differential leucocyte counter. J Clin Pathol 1977;30:997-1004.

26. Carlson DA, Ito RK, Statland BE, Daigneault R, DiPierro R, Horion L. Evaluation of the Sysmex CC-800: an automated eight-parameter hematology instrument. Am J Clin Pathol 1986;86:55-60.

27. Payne BA, Pierre RV, Lee WK. Evaluation of the TOA E-5000 automated hematology analyzer. Am J Clin Pathol 1987;88:51-7.

28. Simmons A, Leaverton P, Elbert G. Normal laboratory values for differential white cell counts established by manual and automated cytochemical methods (Hemalog D). J Clin Pathol 1974;27:55-8.

29. Pinkerton PH, Spence I, Ogilvie JC, Ronald WA, Marchant P, Ray PK. An assessment of the Coulter counter model S. J Clin Pathol 1970;23:68-76.

30. Weiser MG. Comparison of two automated multi-channel blood cell counting systems for analysis of blood of common domestic animals. Vet Clin Pathol 1984;12:25-32.

31. Weiser MG. Modification and evaluation of a multichannel blood cell counting system for blood analysis in veterinary hematology. J Amer Vet Med Assoc 1987;190:411-5.

32. Weiser MG. Size referenced electronic leukocyte counting threshold and lysed leukocyte size distribution of common domestic animal species. Vet Pathol 1987;24:560-3.

33. Kamentsky LA, Melamed MR, Derman H. Spectrophotometer: new instrument for ultrarapid cell analysis. Science 1965;150:630-1.

34. Kamentsky LA, Melamed MR. Spectrophotometric cell sorter. Science 1967;156:1364-5.

35. Fulwyler MJ. Electronic separation of biological cells by volume. Science 1965;150:910-1.

36. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:19-20.

37. Shapiro HM. Multistation multiparameter flow cytometry: a critical review and rationale. Cytometry 1983;3:227-43.

38. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:22.

39. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:31.

40. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:44-48.

41. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:51-53.

42. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:23-24.

43. Loken MR, Stall AM. Flow cytometry as an analytical and preparative tool in immunology. J Immun Methods 1982;50:R85-R112.

44. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:55-57.

45. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:78-79.

46. Bloch D, Beaty N, Fu CT, Chin E, Smith J, Pipkin JL Jr. Flow-cytometric analysis of chicken red blood cells. J Histochem Cytochem 1978;26:170-86.

47. Loken MR, Parks DR, Herzenberg LA. Identification of cell asymmetry and orientation by light scattering. J Histochem Cytochem 1977;25:790-5.

48. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:85-86.

49. Sharpless T, Bartholdi M, Melamed MR. Size and refractive index dependence of simple forward angle scattering measurements in a flow system using sharply-focused illumination. J Histochem Cytochem 1977;25:845-56.

50. Kerker M, Chew H, McNulty PJ, et al. Light scattering and fluorescence by small particles having internal structure. J Histochem Cytochem 1979;27:250-63.

51. Kim YR, Ornstein L. Isovolumetric sphering of erythrocytes for more accurate and precise cell volume measurement by flow cytometry. Cytometry 1983;3:419-27.

52. Steinkamp JA, Crissman HA. Automated analysis of deoxyribonucleic acid, protein and nuclear to cytoplasmic relationships in tumor cells and gynecologic specimens. J Histochem Cytochem 1974;22:616-21.

53. Curbelo R, Schindkraut ER, Hirschfeld T, Webb RH, Block MJ, Shapiro HM. A generalized machine for automated flow cytology system design. J Histochem Cytochem 1976;24:388-95.

54. Shapiro HM. Fluorescent dyes for differential counts by flow cytometry: does histochemistry tell us much more than cell geometry? J Histochem Cytochem 1977;25:976-89.

55. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:87-88.

56. Aubin JE. Autofluorescence of viable cultured mammalian cells. J Histochem Cytochem 1979;27:36-43.

57. Benson PC, Meyer RA, Zaraba ME, McKhann GM. Cellular autofluorescence - is it due to flavins? J Histochem Cytochem 1979;27:44-8.

58. Thorell B. Intracellular red-ox steady states as basis for cell characterization by flow cytometry. Blood Cells 1980;6:745-51.

59. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:89-90.

60. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:93.

61. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:111.

62. Murphy RF, Powers S, Verderame M, Cantor CR, Pollack R. Flow cytofluorometric analysis of insulin binding and internalization by Swiss 3T3 cells. Cytometry 1982;2:402-6.

63. Osband ME, Cohen EB, McCaffrey RP, Shapiro HM. A technique for the flow cytometric analysis of lymphocytes bearing histamine receptors. Blood 1980;56:923-5.

64. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:60-64.

65. Melamed MR, Lindmo T, Mendelsohn ML, eds. Flow Cytometry and Sorting. 2nd ed. New York: Wiley-Liss, Inc., 1990.

66. Shapiro HM. Practical Flow Cytometry. New York: Alan R. Liss, Inc., 1985:165.

67. Kjelsberg CR. Principles of hematologic examination. In: Lee GR, Bithell TC, Foerster J, Athens JW, Lukens JN, eds. Wintrobe's Clinical Hematology. 9th ed. v. 1. Philadelphia: Lea and Febiger, 1993:7-37.

68. Krause JR. Automated differentials in the hematology laboratory. Am J Clin Pathol 1990;93:S11-6.

69. Duncan JR, Prasse KW, eds. Veterinary Laboratory Medicine. 2nd ed. Ames: Iowa State University Press, 1986.

70. Steinkamp JA, Wilson JS, Saunders GC, Stewart CC. Phagocytosis: flow cytometric quantitation with fluorescent microspheres. Science 1982;215:64-5.

71. Salzman GC, Crowell JM, Martin JC, et al. Cell classification by lazer light scattering: identification and separation of unstained leukocytes. Acta Cytol 1975;19:374-7.

72. Shapiro HM, Schildkraut ER, Curbelo R, Laird CW, Turner RB, Hirschfeld T. Combined blood cell counting and classification with fluorochrome stains and flow instrumentation. J Histochem Cytochem 1976;24:396-411.

73. Adams LR, Kamentsky LA. Machine characterization of human leukocytes by acridine orange fluorescence. Acta Cytol 1971;15:289-91.

74. Williams WJ, Nelson DA, Morris MW. Examination of the blood. In: Williams WJ, Beutler E, Ersler AJ, Lichtman MA, eds. Hematology. 4th ed. New York: McGraw-Hill, Inc., 1990:9-24.

75. Tvedten H. Advanced hematology analyzers: interpretation of results. Vet Clin Pathol 1993;22:72-80.

76. England JM, Rowan RM, van Assendelft OW, et al. Protocol for evaluation of automated blood cell counters. Clin Lab Haemat 1984;6:69-84.

77. Gaunt SD, Prescott-Mathews JS, King WW, Scholl DT. Clinical hematology practices at Veterinary teaching hospitals and private diagnostic laboratories. Vet Clin Pathol (in press).

78. DeCresce RD. The CC-800 eight-parameter, fully automated hematology analyzer with autosampler. J Clin Lab Autom 1983;3:375-6.

79. Weingand KW, Odioso LW, Dameron GW, Laytart MJ, Stitzel KA. Hematology analyzer comparison: Ortho ELT-8/ds vs. Baker 9000 for healthy dogs, mice, and rats. Vet Clin Pathol 1992;21:10-4.

80. Yacko M, Swedlund J, Weil SC. Multiple methods for platelet enumeration: observation of a newly introduced bias. Am J Clin Pathol 1987;87:109-12.

81. Bentley SA, Johnson A, Bishop CA. A parallel evaluation of four automated hematology analyzers. Am J Clin Pathol 1993;100:626-32.

82. Tvedten HW, Wilkins RJ. Automated blood cell counting systems: a comparison of the Coulter S-Plus IV, Ortho ELT-8/DS, Ortho ELT-8/WS, Technicon H-A, and Sysmex E-5,000. Vet Clin Pathol 1988;17:47-54.

83. Knoll JS. Comparison of the CDC Mascot and the Serono-Baker 9000 for analysis of the erythrocyte parameters. In: AACC Division of Animal Clinical Chemistry Conference on Automated Hematologic Analysis for Animal Species. Indianapolis, IN:, 1992.

84. Lewis SM, Bentley SA. Haemocytometry by laser-beam optics: evaluation of the Hemac 630L. J Clin Pathol 1977;30:54-64.

85. Breakell ES, Marchand A, Marcus R, Simson E. Comparison of performance for leukocyte differential counting of the Technicon H6000 system with a manual reference method using the NCCLS standard. Blood Cells 1985;11:257-79.

86. Ross DW, Bentley SA. Evaluation of an automated hematology system (Technicon H-1). Arch Pathol Lab Med 1986;110:803-8.

87. DeCresce R. The Technicon H-1: a discrete, fully automated complete blood count and differential analyzer. Lab Med 1986 January;17:17-21.

88. Bollinger PB, Drewinko B, Brailas CD, Smeeton NA, Trujillo JM. The Technicon H-1: an automated hematology analyzer for today and tomorrow. Am J Clin Pathol 1987;87:71-8.

89. Bollinger PB, Drewinko B, Brailas C, Hickey S, Johnston DA, Trujillo JM. A multiparameter automated hematology analyzer: the Technicon H-6000. Lab Med 1987;18:21-7.

90. Cornbleet PJ, Myrick D, Judkins S, Levy R. Evaluation of the Cell-Dyn 3000 differential. Am J Clin Pathol 1992;98:603-14.

91. Tycko DH, Metz MH, Epstein EA, Grinbaum A. Flow-cytometric light scattering measurement of red blood cell volume and hemoglobin concentration. Appl Optics 1985;24:1355-65.

92. Drewinko B, Bollinger P, Rountree M, et al. Eight-parameter automated hematology analyzers: comparison of two flow cytometric systems. Am J Clin Pathol 1982;78:738-47.

93. Wenz B, Gennis P, Canova C, Burns ER. The clinical utility of the leukocyte differential in emergency medicine. Am J Clin Pathol 1986;86:298-303.

94. Lane R. Basic techniques in pet avian clinical pathology. Vet Clin North Am Small Anim Pract 1991;21:1157-79.

95. Lucas AM, Jamroz C, eds. Atlas of Avian Hematology. Agriculture Monograph 25. Washington, D.C.: United States Department of Agriculture, 1961:232.

96. Campbell TW, Dein FJ. Avian hematology: the basics. Vet Clin North Am Small Anim Pract 1984;14:223-48.

97. Natt MP, Herrick CA. A new blood diluent for counting erythrocytes and leucocytes of the chicken. Poult Sci 1952;31:735-8.

98. Shaw AFB. The leucocytes of the pigeon with special reference to a diurnal rhythm. J Pathol Bacteriol 1933;37:411-30.

99. Rees M, Ecker EE. An improved method for counting blood platelets. J Amer Med Assoc 1923;80:621-2.

100. DeEds F. Normal blood counts in pigeons. J Lab Clin Med 1927;12:437-8.

101. Lucas AM, Jamroz C, eds. Atlas of Avian Hematology. Agriculture Monograph 25. Washington, D.C.: United States Department of Agriculture, 1961:231.

102. Dein FJ, ed. Laboratory Manual of Avian Hematology. East Northport: Association of Avian Veterinarians, 1984.

103. Wiseman BK. An improved method for obtaining white cell counts in avian blood. Proc Soc Exp Biol Med 1931;28:1030-3.

104. Lucas AM, Jamroz C, eds. Atlas of Avian Hematology. Agriculture Monograph 25. Washington, D.C.: United States Department of Agriculture, 1961:232-234.

105. Olson C. Avian hematology. In: Biester HE, Swarte LH, eds. Diseases of Poultry. 5th ed. Ames: Iowa State University Press, 1965:100-19.

106. Swayne DE, Stockham SL, Johnson GS. Cytochemical properties of chicken blood cells resembling both thrombocytes and lymphocytes. Vet Clin Pathol 1986;15:17-24.

107. Watt SM, Burgess AW, Metcalf D, Battye FL. Isolation of mouse bone marrow neutrophils by light scatter and autofluorescence. J Histochem Cytochem 1980;28:934-46.

108. Lucas AM, Jamroz C, eds. Atlas of Avian Hematology. Agriculture Monograph 25. Washington, D.C.: United States Department of Agriculture, 1961:212-215.

109. Atwal OS, McFarland LZ. A morphologic and cytochemical study of erythrocytes and leukocytes of *Coturnix coturnix japonica*. Amer J Vet Res 1966;27:1059-65.

110. Topp RC, Carlson HC. Studies on avian heterophils, II. Histochemistry. Avian Dis 1972;16:369-73.

111. Maiti NK, Saini SS, Sharma SN. Histochemical studies on chicken peripheral blood lymphocytes. Vet Res Commun 1990;14:207-10.

112. Thorell B. Flow cytometric analysis of cellular endogenous fluorescence simultaneously with emission from exogenous fluorochromes, light scatter, and absorption. Cytometry 1981;2:39-43.

113. Thorell B. Flow cytometric monitoring of intracellular flavins simultaneously with NAD(P)H levels. Cytometry 1983;4:61-5.

114. Lis M, Julesz J, Gutkowska J, Genest J. Neutrophil activation monitored by flow cytometry: stimulation by phorbol diester is an all-or-none event. Science 1982;215:673-7.

115. Weil GJ, Chused TM. Eosinophil autofluorescence and its use in isolation and analysis of human eosinophils using flow microfluorometry. Blood 1981;57:1099-104.

116. Traganos F, Darzynkiewicz Z, Sharpless T, Melamed MR. Simultaneous staining of ribonucleic and deoxyribonucleic acids in unfixed cells using acridine orange in a flow cytofluorometric system. J Histochem Cytochem 1977;25:46-56.

117. Darzynkiewicz Z, Kapuscinski J. Acridine orange: a versatile probe of nucleic acids and other cell constituents. In: Melamed MR, Lindmo T, Mendelsohn ML, eds. Flow Cytometry and Sorting. 2nd ed. New York, NY: Wiley-Liss, Inc., 1990:291-314.

118. Melamed MR, Adams LR, Traganos F, Kamentsky LA. Blood granulocyte staining with acridine orange changes with infection. J Histochem Cytochem 1974;22:526-30.

119. Steinkamp JA, Fulwyler MJ, Coulter JR, Hiebert RD, Horney JL, Mullaney PF. A new multiparameter separator for microscopic particles and biological cells. Rev Sci Instrum 1973;44:1301-10.

120. Steinkamp JA, Romero A. Identification of discrete classes of normal human peripheral lymphocytes by multiparameter flow analysis. Proc Soc Exp Biol Med 1974;146:1061-6.

121. Crissman HA, Steinkamp JA. Rapid, simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. J Cell Biol 1973;59:766-71.

122. Crissman HA, Oka MS, Steinkamp JA. Rapid staining methods for analysis of deoxyribonucleic acid and protein in mammalian cells. J Histochem Cytochem 1976;24:64-71.

123. Miller JS, Quarles JM. Flow cytometric identification of microorganisms by dual staining with FITC and PI. Cytometry 1990;11:667-75.

124. Pollack A. Flow cytometric cell-kinetic analysis by simultaneously staining nuclei with propidium iodide and fluorescein isothiocyanate. In: Darzynkiewicz Z, Crissman HA, eds. Methods in Cell Biology. v. 33. San Diego: Academic Press, Inc., 1990:315-23.

125. Lee LG, Chen C, Chiu LA. Thiazole orange: a new dye for reticulocyte analysis. Cytometry 1986;7:508-17.

126. Mura C, Huang PC. Immunofluorescent study of histone H5 in chick erythroid cells from developing embryos and adults. Mech Ageing Dev 1978;7:109-22.

127. Zentgraf H, Scheer U, Franke WW. Characterization and localization of the RNA synthesized in mature avian erythrocytes. Exp Cell Res 1975;96:81-95.

128. Lazarides E. From genes to structural morphogenesis: the genesis and epigenesis of a red blood cell. Cell 1987;51:345-56.

129. Marchesi VT, Steers E. Selective solubilization of a protein component of the red cell membrane. Science 1968;159:203-4.

130. Goodman SR, Krebs KE, Whitfield CF, Riederer BM, Zagon IS. Spectrin and related molecules. CRC Crit Rev Biochem 1988;23:171-234.

131. Coleman TR, Fishkind DJ, Mooseker MS, Morrow JS. Functional diversity among spectrin isoforms. Cell Motil Cytoskeleton 1989;12:225-47.

132. Branton D, Cohen CM, Tyler J. Interaction of cytoskeletal proteins on the human erythrocyte membrane. Cell 1981;24:24-32.

133. Burridge K, Kelly T, Mangeat P. Nonerythrocyte spectrins: actin-membrane attachment proteins occurring in many cell types. J Cell Biol 1982;95:478-86.

134. Bennett V. Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. Physiol Rev 1990;70:1029-65.

135. Wasenius VM, Saraste M, Knowles J, Virtanen I, Lehto V-P. Sequencing of the chicken non-erythroid spectrin cDNA reveals an internal repetitive structure homologous to the human erythocyte spectrin. EMBO J 1985;4:1425-30.

136. Lazarides E, Nelson WJ. Erythrocyte and brain forms of spectrin in cerebellum: distinct membrane-cytoskeletal domains in neurons. Science 1983;220:1295-6.

137. Repasky EA, Granger BL, Lazarides E. Widespread occurrence of avian spectrin in nonerythroid cells. Cell 1982;29:821-33.

138. Hiller G, Weber K. Spectrin is absent in various tissue culture cells. Nature 1977;266:181-3.

139. Ngai J, Capetanaki YG, Lazarides E. Differentiation of murine erythroleukemia cells results in the rapid repression of vimentin gene expression. J Cell Biol 1984;99:306-14.

140. Granger BL, Lazarides E. Structural associations of synemin and vimentin filaments in avian erythrocytes revealed by immunoelectron microscopy. Cell 1982;30:263-75.

141. Cohen WD. The cytockeletal system of nucleated erythrocytes. Int Rev Cytol 1991;130:37-84.

142. Geiduschek JB, Singer SJ. Molecular changes in the membranes of mouse erythroid cells accompanying differentiation. Cell 1979;16:149-63.

143. Granger BL, Repasky EA, Lazarides E. Synemin and vimentin are components of intermediate filaments in avian erythrocytes. J Cell Biol 1982;92:299-312.

144. Solomon F, Magendantz M, Salzman A. Identification with cellular microtubules of one of the co-assembling microtubule-associated proteins. Cell 1979;18:431-8.

145. Miller M, Solomon F. Kinetics and intermediates of marginal band reformation: evidence for peripheral determinants of microtubule organization. J Cell Biol 1984;99:70s-5s.

146. Murphy DB, Wallis KT. Isolation of microtubule protein from chicken erythrocytes and determination of the critical concentration for tubulin polymerization *in vitro* and *in vivo*. J Biol Chem 1983;258:8357-64.

147. Maser MD, Philpott CW. Marginal bands in nucleated erythrocytes. Anat Rec 1964;150:365-82.

148. Grecchi R, Saliba AM, Mariano M. Morphological changes, surface receptors and phagocytic potential of fowl mono-nuclear phagocytes and thrombocytes *in vivo* and *in vitro*. J Path 1980;130:23-31.

149. Dhingra LD, Parrish WB, Venzke WG. Electron microscopy of nongranular leukocytes and thrombocytes of chickens. Amer J Vet Res 1969;30:1837-42.

150. Maxwell MH, Trejo F. The ultrastructure of white blood cells and thrombocytes of the domestic fowl. Br Vet J 1970;126:583-92.

151. Murphy DB, Grasser WA, Wallis KT. Immunofluorescence examination of beta tubulin expression and marginal band formation in developing chicken erythroblasts. J Cell Biol 1986;102:628-35.

152. Wang D, Villasante A, Lewis SA, Cowan NJ. The mammalian β-tubulin repertoire: hematopoietic expression of a novel, heterologous β-tubulin isotype. J Cell Biol 1986;103:1903-10.

153. Grasso JA. Cytoplasmic microtubules in mammalian erythropoietic cells. Anat Rec 1966;156:397-414.

154. van Deurs B, Behnke O. The microtubule marginal band of mammalian red blood cells. Z Anat Entwickl-Gesch 1973;143:43-7.

155. Davis BH, Bigelow NC. Flow cytometric reticulocyte quantification using thiazole orange provides clinically useful reticulocyte maturity index. Arch Pathol Lab Med 1989;113:684-9.

156. Barclay NE. Marginal bands in duck and camel erythrocytes. Anat Rec 1966;154:313.

157. Joseph-Silverstein J, Cohen WD. The cytoskeletal system of nucleated erythrocytes. III. Marginal band function in mature cells. J Cell Biol 1984;98:2118-25.

158. Cohen WD. Observations on the marginal band system of nucleated erythrocytes. J Cell Biol 1978;78:260-73.

159. Bryan J, Wilson L. Are cytoplasmic microtubules heteropolymers? Proc Nat Acad Sci USA 1971;68:1762-6.

160. Kim S, Magendantz M, Katz W, Solomon F. Development of a differentiated microtubule structure: formation of the chicken erythrocyte marginal band in vivo. J Cell Biol 1987;104:51-9.

161. Jennette JC, Wick MR. Immunohistochemical techniques. In: Jennette JC, ed. Immunohistology in Diagnostic Pathology. Boca Raton: CRC Press, Inc., 1989:1-28.

162. Osborn M, Weber K. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures. In: Wilson L, ed. Methods in Cell Biology. v. 24. New York: Academic Press, Inc., 1982:97-132.

163. Sternberger LA. Immunocytochemistry. 3rd ed. New York: John Wiley and Sons, Inc., 1986:43.

164. Lucas AM, Jamroz C, eds. Atlas of Avian Hematology. Agriculture Monograph 25. Washington, D.C.: United States Department of Agriculture, 1961:216.

165. Waggoner AS. Fluorescent probes for cytometry. In: Melamed MR, Lindmo T, Mendelsohn ML, eds. Flow Cytometry and Sorting. 2nd ed. New York: Wiley-Liss, Inc., 1990:209-25.

166. Fothergill JE. Fluorochromes and their conjugation with proteins. In: Nairn RC, ed. Fluorescent Protein Tracing. 3rd ed. Baltimore: The Williams and Wilkins Company, 1969:5-34.

167. Dyson JED, McLaughlin JB, Surrey CR, Simmons DM, Daniel J. Effects of hyperthermia, irradiation, and cytotoxic drugs on fluorescein isothiocyanate staining intensity for flow cytometry. Cytometry 1987;8:26-34.

168. Campbell TW. Hematology of birds, reptiles, and fish. Vet Techn 1990;11:538-42.

169. Jain NC. Osmotic fragility of erythrocytes of dogs and cats in health and in certain hematologic disorders. Cornell Vet 1973;63:411-23.

170. Viscor G, Palomeque J. Method for determing the osmotic fragility curves of erythrocytes in birds. Lab Anim 1982;16:48-50.

171. Chang CF, Hamilton PB. The thrombocyte as the primary phagocyte in chickens. J Reticuloendothel Soc 1979;25:585-90.

172. Loken MR, Herzenberg LA. Analysis of cell populations with a fluorescence-activated cell sorter. Ann N Y Acad Sci 1975;254:163-71.

173. Shapiro HM. Flow cytometric estimation of DNA and RNA content in intact cells stained with Hoechst 33342 and pyronin Y. Cytometry 1981;2:143-50.

174. Rotman B, Papermaster BW. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc Nat Acad Sci USA 1966;55:134-41.

175. Rotman B, Zderic JA, Edelstein M. Fluorogenic substrates for β-D-galactosidases and phosphatases derived from fluorescein (3,6-dihydrofluoran) and its monomethyl ether. Proc Nat Acad Sci USA 1963;50:1-6.

176. Watson JV. Enzyme kinetic studies in cell populations using fluorogenic substrates and flow cytometric techniques. Cytometry 1980;1:143-51.

177. LePecq JB, Paoletti C. A new fluorometric method for RNA and DNA determination. Analyt Biochem 1966;17:100-7.

178. Hudson B, Upholt WB, Devinny J, Vinograd J. The use of an ethidium analogue in the dye-buoyant density procedure for the isolation of closed circular DNA: the variation of the superhelix density of mitochondrial DNA. Proc Nat Acad Sci USA 1969;62:813-20.

179. Lakhanpal S, Gonchoroff NJ, Katzmann JA, Handwerger BS. A flow cytofluorometric double staining technique for simultaneous determination of human mononuclear cell surface phenotype and cell cycle phase. J Immun Methods 1987;96:35-40.

180. LePecq JB. Use of ethidium bromide for separation and determination of nucleic acids of various conformational forms and measurement of their associated enzymes. In: Glick D, ed. Methods of Biochemical Analysis. v. 20. New York: John Wiley and Sons, Inc., 1971:41-86.

181. LePecq JB, Paoletti C. A fluorescent complex between ethidium bromide and nucleic acids. J Mol Biol 1967;27:87-106.

182. Krishan A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 1975;66:188-93.

183. George LS, Dallas CE, Brisbin IL, Evans DL. Flow cytometric DNA analysis of ducks accumulating ^{137}Cs on a reactor reservoir. Ecotoxicol Environ Safety 1991;21:337-47.

184. Schmitz FJ, Werner E. Optimization of flow-cytometric discrimination between reticulocytes and erythrocytes. Cytometry 1986;7:439-44.

185. Reagan WJ, Vap LM, Weiser MG. Flow cytometric analysis of feline reticulocytes. Vet Pathol 1992;29:503-8.

186. Abbott DL, McGrath JP. Evaluation of flow cytometric counting procedure for canine reticulocytes by use of thiazole orange. Amer J Vet Res 1991;52:723-7.

187. Lucas AM, Jamroz C, eds. Atlas of Avian Hematology. Agriculture Monograph 25. Washington, D.C.: United States Department of Agriculture, 1961:27.

188. Glick B, Madyastha P, Koger B, LaVia MF. The use of Ficoll-Hypaque double density gradients in the separation of avian granulocytes from other cell types for the purpose of cell flow cytometric analysis. Dev Comp Immunol 1985;9:477-84.

189. Zinkl JG. Avian hematology. In: Jain NC, ed. Schalm's Veterinary Hematology. 4th ed. Philadelphia: Lea and Febiger, 1986:256-73.

190. Loken MR, Stout RD, Herzenberg LA. Lymphoid cell analysis and sorting. In: Melamed MR, Mullaney PF, Mendelsohn ML, eds. Flow Cytometry and Sorting. New York: John Wiley and Sons, 1979:505-28.

191. Laerum OD, Farsund T. Clinical application of flow cytometry: a review. Cytometry 1981;2:1-13.

192. Kung PC, Goldstein G, Reinherz EL, Schlossman SF. Monoclonal antibodies defining distinctive human T cell surface antigens. Science 1979;206:347-9.

193. Hoffman RA, Kung PC, Hansen WP, Goldstein G. Simple and rapid measurement of human T lymphocytes and their subclasses in peripheral blood. Proc Nat Acad Sci USA 1980;77:4914-7.

194. Sallan SE, Ritz J, Pesando J, et al. Cell surface antigens: prognostic implications in childhood acute lymphoblastic leukemia. Blood 1980;55:395-402.

195. Ligler FS, Smith RG, Kettman JR, et al. Detection of tumor cells in the peripheral blood of nonleukemic patients with B-cell lymphoma: analysis of "clonal excess". Blood 1980;55:792-801.

196. Brown G, Cappallaro D, Greaves M. Leukemia-associated antigens in man. J Natl Cancer Inst 1975;55:1281-9.

197. Loike JD, Silverstein SC. A fluorescence quenching technique using trypan blue to differentiate between attached and ingested glutaraldehyde-fixed red blood cells in phagocytosing murine macrophages. J Immun Methods 1983;57:373-9.

198. Gozes I, Barnstable CJ. Monoclonal antibodies that recognize discrete forms of tubulin. Proc Nat Acad Sci USA 1982;79:2579-83.

199. Henderson C. Aminoalkylsilane: an inexpensive, simple preparation for slide adhesion. J Histotechnol 1989;12:123-4.

200. Hsu S-M, Raine L, Fanger H. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PA) procedures. J Histochem Cytochem 1981;29:577-80.

201. Cordell JL, Falini B, Erber WN, et al. Immunoenzymatic labeling of monoclonal antibody using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatse (APAAP complex). J Histochem Cytochem 1984;32:219-29.

202. Jacobberger JW, Fogleman D, Lehman JM. Analysis of intracellular antigens by flow cytometry. Cytometry 1986;7:356-64.

203. Osborn M, Franke WW, Weber K. Visualization of a system of filaments 7-10 nm thick in cultured cells of an epithelioid line (Pt K2) by immunofluorescence microscopy. Proc Nat Acad Sci USA 1977;74:2490-4.

204. Connolly JA, Kalnins VL. Visualization of centrioles and basal bodies by fluorescent staining with non-immune rabbit serum. J Cell Biol 1978;79:526-32.

205. Gordon WE III, Bushnell A, Burridge K. Characterization of the intermediate (10 nm) filaments of cultured cells using an autoimmune rabbit antiserum. Cell 1978;13:249-61.

206. Sternberger LA. Immunocytochemistry. 3rd ed. New York: John Wiley and Sons, Inc., 1986:52.

207. Sternberger LA. Immunocytochemistry. 3rd ed. New York: John Wiley and Sons, Inc., 1986:49.

208. Ottesen EA, Stanley AM, Gelfand JA, et al. Immunoglobulin and complement receptors on human eosinophils and their role in cellular adherence to schistosomules. Am J Trop Med Hyg 1977;26:134-41.

209. Sternberger LA. Immunocytochemistry. 3rd ed. New York: John Wiley and Sons, Inc., 1986:51.

210. Floyd K, Suter PF, Lutz H. Granules of blood eosinophils are stained directly by anti-immunoglobulin fluorescein isothiocyanate conjugates. Amer J Vet Res 1983;44:2060-3.

211. Sternberger LA. Immunocytochemistry. 3rd ed. New York: John Wiley and Sons, Inc., 1986:50.

212. Valnes K, Brandtzaeg P. Selective inhibition of nonspecific eosinophil staining or identification of eosinophilic granulocytes by paired counterstaining in immunofluorescence studies. J Histochem Cytochem 1981;29:595-600.

213. Valnes K, Brandtzaeg P. Unlabeled antibody peroxidase-anti-peroxidase method combined with direct immunofluorescence. J Histochem Cytochem 1981;29:703-11.

214. Johnston N, Bienenstock J. Abolition on non-specific fluorescent staining of eosinophils. J Immun Methods 1974;4:189-94.

215. Crissman HA, Mullaney PF, Steinkamp JA. Methods and applications of flow systems for analysis and sorting of mammalian cells. In: Prescott DM, ed. Methods in Cell Biology. v. 9. New York, NY: Academic Press, Inc., 1975:179-246.

216. Ledbetter JA, Rouse RV, Micklem HS, Herzenberg LA. T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. J Exp Med 1980;152:280-95.

217. Franke WW, Seib E, Osborn M, Weber K, Herth W, Falk H. Tubulin-containing structures in the anastral mitotic apparatus of endosperm cells of the plant Leucojun aestivum as revealed by immunofluorescence microscopy. Cytobiologie 1977;15:24-45.

218. Weber K, Osborn M, Franke WW, Seib E, Scheer U, Herth W. Identification of microtubular structures in diverse plant and animal cells by immunological cross-reaction revealed in immunofluorescence microscopy using antibody against tubulin from porcine brain. Cytobiologie 1977;15:285-302.

219. Lloyd CW, Slabas AR, Powell AJ, MacDonald G, Badley RA. Cytoplasmic microtubules of higher plant cells visualized with anti-tubulin antibodies. Nature 1979;279:239-41.

220. Cleveland DW, Sullivan KF. Molecular biology and genetics of tubulin. Ann Rev Biochem 1985;54:331-65.

221. Birgbauer E, Solomon F. A marginal band-associated protein has properties of both microtubule- and microfilament-associated proteins. J Cell Biol 1989;109:1609-20.

222. Jackson RC. The exterior surface of the chicken erythrocyte. J Biol Chem 1975;250:617-22.

223. Wiese MJ, Ingram VM. Proteins and glycoproteins of membranes from developing chick red cells. J Biol Chem 1976;25:6667-73.

224. Fulton JE, Hall VJ, Lamont SJ. Ontogeny and expression of chicken A blood group antigens. Anim Genet 1990;21:47-57.

225. Schierman LW, Nordskog AW. Relationship of blood type to histocompatibilty in chickens. Science 1961;134:1008-9.

226. Fulton JE, Briles WE, Lamont SJ. Chicken A blood group system antigens: molecular characteristics and lack of expression on lymphocytes. Anim Genet 1990;21:401-10.

227. Janzarik H, Schauenstein K, Wolf H, Wick G. Antigenic surface determinants of chicken thrombocytoid cells. Dev Comp Immunol 1980;4:123-35.

228. Péault B, Coltey M, Le Douarin NM. Ontogenic emergence of a quail leukocyte/endothelium cell surface antigen. Cell Differ 1988;23:165-74.

229. Lillehoj HS, Lillehoj EP, Weinstock D, Schat KA. Functional and biochemical characterizations of avian T lymphocytes antigens identified by monoclonal antibodies. Eur J Immunol 1988;18:2059-65.

230. Traill KN, Böck G, Boyd RL, Ratheiser K, Wick G. Ontogeny of surface markers on functionally distinct T cell subsets in the chicken. Eur J Immunol 1984;14:16-67.

231. Paramithiotis E, Tkalec L, Ratcliffe JH. High levels of CD45 are coordinately expressed with CD4 and CD8 on avian thymocytes. J Immunol 1991;147:3710-7.

Vita

William Weaver King was born on May 20, 1965 in Shreveport, Louisiana to Jana Weaver King and William Douglas King, M.D. He graduated from First Baptist Church High School in Shreveport and continued his education at Rhodes College in Memphis, Tennessee. In Memphis, he developed an interest in Veterinary Medicine and research by volunteering in both the Laboratory Animal facility at Rhodes and the Aviary at the Overton Park Zoo and Aquarium. He graduated *cum laude* from Rhodes in 1987 with a Bachelor of Science degree in Biology, whereupon he was accepted in Veterinary School at Louisiana State University in Baton Rouge.

His pursuit of a profession in non-traditional Veterinary Medicine led William to serve as Chair of the Louisiana State University Raptor and Wildlife Rehabilitation Unit as a Veterinary student. Primarily through the guidance of Dr. W. Sheldon Bivin, this interest evolved into a Residency in Laboratory Animal Medicine following graduation from Veterinary School in 1991. The studies began as a Resident were then continued as a Graduate Assistance in pursuit of a Doctor of Philosophy degree in Veterinary Clinical Sciences through the Department of Veterinary Pathology.

William has the distinct honor of being married to Catherine Phister King, with whom he shares the pleasure of parenting William Douglas King, II. They currently reside in Hammond, Louisiana, with their housemate, confidante, and occasional feline pet, Rutabaga.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: William W. King

Major Field: Veterinary Medical Sciences

Title of Dissertation: Flow Cytometric Analysis of Avian Blood Cells: Differentiation of Erythrocytes and Leukocytes by Fluorescence

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

July 10, 1995