CHAPTER 1: INTRODUCTION

1.1 DIFFERENTIAL LEUKOCYTE COUNT

1.1.1 Introduction

The Leukocyte Differential Count is the determination of the proportion of, or absolute count per unit volume of defined classes or subsets of leukocytes in a blood sample[41, 42, 51]. The purpose of the differential count is to obtain a picture of the true distribution of the leukocytes in peripheral blood[3]. This is used by clinicians to determine the disease status of their patients. The leukocytes present in the peripheral blood are composed of five types of mature cells, i.e. neutrophils, lymphocytes, monocytes, eosinophils and basophils. Subdifferentiation of neutrophils into segmented or mature and band or immature forms and lymphocytes into normal, reactive or blast transformed forms is often done[43, 51]. A category of "other cells" is also sometimes included for all the other nucleated cells found in peripheral blood[51].

Definitions for "differential leukocyte count" vary according to the methods used to identify the leukocyte subclasses[51]. When cells are classified according to their morphological appearance on stained smears, there are different levels of discrimination. At the basic level, "polymorphonuclear" and "mononuclear" (or "round cells") are distinguished. At the intermediate level neutrophils, eosinophils, basophils, monocytes and lymphocytes are identified. At the highest level neutrophils are divided into segmented and band forms and lymphocytes into normal and blast transformed cells[43]. When the cells are identified by electronic counters, there again, are different levels of discrimination. Three-part automated differential analyzers identify major leukocyte groups; i.e. granulocytes, lymphocytes and monocytes[51, 52] or small cells, medium sized cells and large cells[15, 68]. The newer, more sophisticated haematology analyzers give five-

part differential counts (i.e. neutrophils, lymphocytes, monocytes, eosinophils and basophils) with comments on subclasses of neutrophils and lymphocytes as well as "other cells" in the sample[45, 51, 66, 75, 80]. Some of the most advanced analyzers will give a six or seven-part differential count (including band neutrophils and variant lymphocytes into their differentiation), such as the Technicon H-1/H-2/H-3 instruments[45] and the Cell-Dyn 3500 with the latest software package[1].

The techniques used to obtain a differential leukocyte count can be divided into two main groups: manual differential counting and automated differential counting. The manual differential count was introduced by Paul Ehrlich[8] in the early twentieth century and has since maintained a reputation as a valuable routine test amongst clinicians[28]. Attempts to automate differential leukocyte counts have been made since the early 1960's[45, 69] and automated leukocyte counts are currently widely used.

1.1.2 Manual Differential Leukocyte Counting

A manual differential leukocyte count is obtained through the morphological evaluation and identification of leukocytes on a blood film, stained with a Romanowsky stain and examined with a light microscope. The count is preferably conducted using the 1000 X magnification under oil immersion[41], but it can also be performed under lower magnifications (400 X and 500 X magnifications) by experienced personnel.

Various methods for the performance of a manual differential leukocyte count have been described. There are variations in the method used to collect the blood, the technique used to make the smear, the pattern used to count the cells and the number of cells counted. The mere fact that there is so much variation in the techniques, should indicate that results cannot be compared reliably unless a standardized method is used.

Factors that may influence the results of the leukocyte differential count when collecting the blood are:

- The physiological state of the patient at the time of collection[47]. Various studies have shown variation in differential counts following a change in climatic conditions. Some studies described differences in samples taken from patients in upright and recumbent positions (orthostatic effect), but others have found this not to be a significant difference[47]. It has also been shown that there is significant diurnal variation in white blood cell counts[8]. Sympathetic stimulation, especially in animals can also play an important role.
- The site of blood collection, i.e. peripheral or venous samples[47].

Blood smears can be made by either using two slides, where one slide is used as a spreader slide, or by using two cover-slides and pulling them apart. Various sizes of blood smears can also be used[47]. Different Romanowsky stains can be used for staining the smears. Vital stains and counting of the leukocytes by a haemocytometer can also be done, but is no longer in general use[47].

The most accurate counting method is to count all the leukocytes on a blood smear. This is obviously a very laborious method that leads to fatigue of the person performing the count. Some studies claimed that the most uniform results are obtained when every leukocyte on a very small film is counted[47]. The reason for the superior results obtained by counting the whole smear is because the white cells do not distribute evenly when a blood smear is made. The uneven distribution of the leukocytes was described and shown not to be accidental, but that the factors influencing the distribution could not be sufficiently controlled for rules to be laid down[47].

The different search patterns used to perform the counts can lead to great variation in the counts[47]. The count can be made by random selection of fields in different parts of the smear, or by a fixed pattern. The methods most commonly used are the straight edge method, the battlement method and the cross-sectional count[40, 47]. In a study done in 1940 these three methods in were compared and the battlement method was identified as the method giving the most accurate results[47]. It is also the method recommended by the National Committee for Clinical Laboratory Standards[51].

The battlement method was described as a "count made of three horizontal edge fields followed by two fields towards the center (so as to give three vertical fields), followed by two fields in a horizontal and then two fields in the vertical direction again"[47]. The straight edge count is performed by identifying and counting the cells in the field adjacent to the edge. Fields along both edges are examined[47]. The cross-sectional method starts at an edge field, moves across the body of the smear to the opposite edge and back across in a similar manner until the desired number of cells have been identified[47].

The number of cells counted when performing a differential leukocyte count varies greatly. The 100-cell differential count is the most common method, because it is the least labor-intensive method. However, it has been shown over and over to be an imprecise method[3, 42, 43, 47, 49]. The differential count does not appear to be sensitive enough to base decisions for the clinical care of patients on, except for the total neutrophil count[43].

One study conducted, indicated that a minimum number of 300 cells should be counted[47]. Various other investigators concurred[47].

The error due to chance is responsible for a large degree of inconsistency in differential leukocyte counts[3]. An error due entirely to chance occurs when our estimate of different types of units in a universe composed of a very large number of individual units is based on a study of an extremely small proportion of them[3]. If the white cell count is 10.0 X $10^9/\ell$, there will be 50 000 000 000 cells in 5 liters of blood, and we count only a few hundred of these![3] Thus even when there is no error in technique or interpretation, the chance error is unavoidable and will still be present[3]. In a similar statistical analysis of leukocyte differential counts, a similar conclusion was reached[42]. It was showed that in a perfect smear, containing exactly 20% lymphocytes, exactly 20% lymphocytes were only counted 9.93% of the time if 100-cell differential counts are done[42]. The error is inversely proportional to the square root of the number of cells counted[3]. Thus it was concluded that at least 400 cells must be counted to obtain reliable results[3]. The National

Committee for Clinical Laboratory Standards[51] also recommends a 400-cell differential count.

The manual differential count is also a very slow and labor-intensive procedure[8, 28, 47]. In a discussion, reported in a paper by Brecher *et al*[8], a Dr Bull comments that it takes 95 seconds to complete a differential count, but he goes on to say that it would be inappropriate to have a technologist do only white blood cell differentials for a whole day. In the experience of the Clinical Pathology Laboratory of the Onderstepoort Veterinary Academic Hospital, one technologist can do approximately thirty accurate manual 100-cell differential counts per day on diseased patients. This is because more thorough examinations of the blood smears have to be done for leukocyte abnormalities and parasites as well as for erythrocyte abnormalities and parasites.

1.1.3 Automated Methods for Differential Leukocyte Counting

Automated differential leukocyte counting methods have various advantages over manual methods. Firstly, most automated instruments count thousands of cells[1, 7, 50] compared to the 100 to 400 cells of the manual methods. This leads to considerable improvement in the precision of the method[50, 51] because the error due to chance is much smaller. Another advantage is the rapid screening of multiple samples, leading to improved efficiency[50]. Some of the modern automated analyzers can process up to 120 samples per hour[45]. A further advantage, is the improved safety that automated analyzers provide, because laboratory staff has less direct contact with the sample and very small sample volumes are required.

Various methods have been employed in the attempt to automate differential leukocyte counting and tremendous progress has been made in the past two decades. Some of the methods had fallen into disuse, only to be improved and introduced at a later stage again, such as quantitative buffy coat analysis and cell pattern recognition.

The methods used in automated leukocyte differential counting are electronic impedance counting and sizing, quantitative buffy coat analysis, cell pattern recognition, flow cytometry, electromagnetic conductivity and various combinations of these techniques.

1.1.3.1 Electronic Impedance Cell Counting and Sizing

Coulter Electronics initially introduced electronic impedance counting in the late 1950's in their Coulter haematology analyzers for cell counting[57]. The method was initially developed to replace manual cell counting, using a haemocytometer, with a faster and more accurate method[48, 57]. However, apart from the number of cells, the size distribution of the cells can also be determined using this method[48].

The blood sample is diluted and the cells are suspended in an electrically conductive medium. The diluted sample is now aspirated through an aperture, which is situated between two electrodes. As the cells pass through the aperture, they cause resistance in the electric circuit and a change in voltage results. This voltage change is then amplified and is shown on an oscilloscope. By using a threshold control circuit only pulses of a selected magnitude range are counted. The amplitude of the pulse is proportional to the cell size and the number of pulses is proportional to the number of cells passing through the aperture[35, 48, 57].

The impedance counters used for differential leukocyte counting generate a three-part differential count. Either the size of the cell or of the cell nucleus is used as the differentiating characteristic. The SYSMEX E-4000 and SYSMEX E-5000 classify the cells as small cells, intermediate sized cells and large[15, 28, 68]. The Coulter Counters, using impedance counting for white cell differentiation classify the cells as lymphocytes, granulocytes and mononuclear cells[28]. The Serono-Baker SYSTEM 9000 calls these subpopulations lymphocytes, midrange cells and granulocytes. Essentially, the small cells are lymphocytes, the intermediate sized cells are mononuclear cells (nuclear size is the discriminating factor) but can also include eosinophils and basophils and the large cells or granulocytes are mainly neutrophils, including band cells[64].

Various reports in the human literature claim that the three-part differentials are sufficient and acceptable for the majority of cases presented for differential counting[2, 15, 28, 68]. Duncan and Gottfried evaluated the usefulness of the three-part differential count in a hospital setting[28]. They found that combined with a qualitative slide review, the three-part differential was suitable for 84% of the samples submitted for a differential count.

1.1.3.2 Quantitative Buffy Coat Analysis

In 1840 Thomas Addison discovered that leukocytes collect above the packed cells when centrifuged. In 1890 Hedin and in the 1930's Wintrobe described the use of the thickness of the buffy coat as a rough estimate of the total white cell count[21]. In 1940 Bessis subdivided the leukocytes in the buffy coat layer from top to bottom as lymphocytes, monocytes and granulocytes[21]. This characteristic of the white blood cells based on their different specific gravities has since been used, together with their different staining reactions with supravital stains, in Quantitative Buffy Coat (QBC) systems to obtain a differential leukocyte count[46,60].

Blood is aspirated into a microtube coated with potassium oxalate and acridine orange[46, 60]. A precision molded cylindrical float is inserted into the microtube before centrifugation[60]. The cylindrical float has a specific gravity of 1.055[46], which is about halfway between that of plasma and the red blood cells. The tube is centrifuged for 5 minutes at 12 000 rpm[46], after which the float settles in the buffy coat layer. The buffy coat length is expanded vertically by a factor of ten[46, 60] because the float occupies 90% of the cross sectional area of the tube[46].

The various components of the buffy coat are further enhanced by the acridine orange[46, 60]. The cells fluoresce differently under excitation of blue violet light. The platelets appear pale yellow, the lymphocytes and monocytes are brilliant green and the granulocytes are orange-yellow[60]. Eosinophils are visible as an

orange-green band at the top of the granulocyte layer in canine and feline samples if the eosinophil count is in excess of 1.0 x $10^9/\ell$[46].

The potassium oxalate prevents the lighter erythrocytes from mixing with the granulocytes[46, 60]. Potassium oxalate removes water from the erythrocytes, causing them to become more dense and smaller and therefore less likely to be present in the granulocyte layer. Anti-red blood cell antibodies have also been used to coat the QBC tubes in order to improve the separation between the red blood cells and the granulocytes[11].

An electronic ocular micrometer using a blue-violet light source[11, 46, 60] measures the lengths of the different layers. Band lengths are given in electronic counts and are then converted to numerical counts[46]. Conversion factors for dogs, cats and horses are available for use in veterinary medicine[46].

In the literature on human haematology QBC analysis is reported to be comparable in clinical sensitivity to the reference method[60]. It is also reported as a rapid, economical haematological evaluation technique in veterinary literature[11, 46] and as a very sensitive method in detecting microfilaraemia[11]. In a recent evaluation of the QBC VetAutoread, it was found to be good for white blood cell counts in dogs, but it was only fair in it's identification of granulocytes and mononuclear cells[53]. This was mainly attributed to insufficient separation of red blood cells and granulocyte layers[53].

1.1.3.3 Cell Pattern Recognition

Digital image analysis as applied to haematology has a long and uncertain history. Red cell counting was automated in the 1950's using television microscopes, but was replaced by flow cell counters[56]. Automated microscopes for haematology were sold in the 1970's[56]. These pattern recognition systems included the Corning LARC Classifier, Geometric Data Hematrak, the Abbott ADC500 and the Coulter Diff3 System[20]. Unfortunately these instruments were not economically

successful and production of most of them has been stopped[56]. However, an improved Artificial Intelligent Diagnostic System has recently been developed by Beksaç *et al*[4], based on a better software algorithm and made possible by the massive improvement in computer processing power in recent years.

Computerized microscopes form the basis of Pattern Recognition Analyzers. Differential leukocyte counts are performed by processing the digital images and classifying them according to their characteristic morphological features[55]. Morphological features evaluated to classify cells include cell area, cell colour, ratio of cell area to circumference, homogeneity of the cell, nuclear area, nuclear colour, homogeneity of the nucleus, cytoplasmic colour etc[4, 20, 55]. The cell identification is thus done in much the same way as a technologist would do it. It is therefore not surprising that cell pattern recognition correlates well with the 100 cell manual differential count, if it also evaluates 100 cells[55, 58, 59]. The biggest advantage of these systems is that 200-, 400-, 600- and 800-cell differentials can easily be done on them because of their increased speed[58]. When the number of cells counted is increased, the chances of detecting abnormal cells will increase. It has been shown that this is indeed the case with the HEMATRAK® [58].

The biggest disadvantage of the pattern recognition systems is that a stained slide is evaluated with a limited number of cells on the slide, often not more than 600. Staining of slides may also provide problems, since the intensity of stains may differ[20].

1.1.3.4 Electromagnetic Conductivity

High-frequency electromagnetic conductivity is only used as part of a combination analysis in the Coulter STKS[70, 45]. The electromagnetic energy is used to determine nuclear size and density[70]. As far as the author is aware its usefulness in the veterinary field has not been evaluated as such.

1.1.3.5 Flow Cytometry

Flow cytometry is a process in which individual cells or other biological particles are made to flow in a single file in a fluid stream past a sensor or sensors that measure the physical or chemical characteristics of the cells or particles[65]. The two techniques used most commonly in combination with flow cytometry for cell counting and identification are flow cytochemistry and laser light scatter measurements. These techniques are often used in combination for the identification of leukocytes.

i) *Flow cytochemistry*

Cytochemistry makes use of the biochemical characteristics of cells[40] and more specifically their staining ability with different dyes. Flow cytochemistry makes use of light scattering and light absorption[33] of the stained cells as they pass the sensor. Various stains can be used, including peroxidase[40]. In flow cytochemistry fluorescent dyes (fluorochromes) are commonly used[33]. One of the most widely used automated leukocyte differential cell counters, the Technicon H*1makes use of flow cytometry with myeloperoxidase[72] as a stain in order to generate a differential leukocyte count. The cells are identified using a combination of their staining reaction and their size[22]. Neutrophils have a strong peroxidase activity; monocytes show a weak activity and lymphocytes have no activity, while eosinophils show intense peroxidase activity[22].

ii) *LASER light scatter*

In 1968 Wyatt[83] explored the possibility that differential light scattering can be used to identify living bacterial cells. His theory was that "the characteristic of each distinct microorganism that scatters light is an essentially unique scattering pattern"[83]. This is due to the unique structural and biochemical features of each type of microorganism. In 1972 Brunsting and Mullaney[13] described light scattering patterns from coated spheres as a model for light scattering from biological cells. They observed that the scattering pattern of spheres at larger

angles were structured and sensitive to the core sphere size. This suggested that it could be used as a possible method for differentiating between biological cells, which are similar in size, but different in internal structure[13]. Later the same year, they described a possible method of mammalian cell identification by differential light scattering[14]. They concluded that scattering measurements made in the forward angle is an indication of cell size, whereas measurements made outside the forward angle are influenced by nuclear size and internal structure[14].

In 1975 Salzman *et al*[61] described a technique using the light scatter at two different angles with respect to the laser beam to identify unstained white blood cells. They were able to differentiate between human lymphocytes, monocytes and granulocytes using this technique. Two benefits of this technique, pointed out by them, are that staining and fixation are not necessary and therefore the risk of introduction of artifacts is limited and secondly very little sample preparation is required[61].

In a further improvement of the technique, De Grooth *et al*[24] introduced the use of a polarized laser beam. Depolarized orthogonal light scatter is measured and it has been shown that the depolarization is caused by multiple scattering processes inside the cell[24]. By using a combination of normal orthogonal light scatter and depolarized orthogonal light scatter, two populations of granulocytes could be identified. Cell sorting was used to demonstrate that the cells with higher depolarization are eosinophils[24]. Terstappen, De Grooth and others then went on to develop a four-parameter leukocyte differential count[69]. They used four different measurements in order to achieve this, namely the intensity of forward angle (0° to 2.6°) scatter, narrow angle (3.0° to 11.0°) scatter and normal and depolarized orthogonal light scatter[69]. The forward angle and the narrow angle scatter are used to differentiate between granulocytes, lymphocytes and monocytes, while the normal and depolarized orthogonal light scatter is used to distinguish between eosinophils and neutrophils[69]. Breaks in the light are used to count the cells[70].

Some of the haematology analyzers using the laser light scatter to count and identify cells include the Ortho ELT-8/ds analyser[78], the Coulter STKS[45, 75] and the Cell-Dyn analyzer range[1, 52, 74].

1.1.3.6 Combinations

Modern haematology analyzers make use of combinations of these techniques to yield accurate and complex multi-parameter counts. The Coulter STKS, for example, makes use of the impedance principle, electromagnetic conductivity and laser light scatter[39, 45, 70]. The Sysmex NE-8000 makes use of a non-optical approach by combining high-energy radio frequency and direct current methodology[39, 45]. The analysis on the Cobas Argos 5-Diff is a combination of impedance-, cytochemistry- and optical absorbance technology[45]. The Cell-Dyn series makes use of the impedance principle and laser light scatter by regular and depolarized light[1, 26, 31, 45, 74].

1.1.3.7 Use of Automated Methods in the Veterinary Field

Haematology analyzers are developed for use in the human medical field. As with other modern technological developments in medical science, it is inevitable that it would be applied to veterinary science. This should be done with caution and only after the specific analyzer has been evaluated for its use in the veterinary field. It is only in recent years that some of the manufacturers of haematology analyzers have started to consider the veterinary side as a potential market and are now adapting their instruments for use in the veterinary field.

The evaluation of animal leukocytes on analyzers developed for the human medical market has a number of potential problem areas. The first problem is the leukocyte size variation between different species[79, 81] and between animal and human leukocytes. When using impedance counting methods, it is important to set the lower threshold carefully in order to minimize the error associated with the interference of debris[79].

The second problem is that the staining reactions of leukocytes differ between species. This creates problems in systems using flow cytochemistry, such as the Technicon H-1, H-2 and H-3 analyzers. The Technicon H-1 has been evaluated and found acceptable for the determination of differential leukocyte counts in dogs[22, 71, 73] since the staining reactions of canine leukocytes are similar to those of human leukocytes. However, it has been found that the equine leukocyte differential count on the H-1 was unacceptable and the reason given for this, is that equine leukocytes, and specifically neutrophils, stain more weakly with peroxidase than human leukocytes[72, 73].

Another potential problem includes the difference between human and animal granulocyte granules in systems making use of the characteristics of the granules to differentiate between cell types. The difference in the reaction of cells to lytic reagents[25, 26, 54, 79] in systems making use of the principle of lysis to either eliminate certain cells, such as erythrocytes, or to strip the leukocyte cytoplasm in order to evaluate the nuclei can also possibly lead to errors. It is thus very important to evaluate an instrument for the acceptable use in every species that would be analyzed on it.

A number of electronic cell counters have recently been evaluated for their use in the veterinary field. When the results of these studies are analyzed, the importance of prior evaluation are clearly illustrated. Green reported on the effect that platelets sometimes have on red cell indices when using the Cell-Dyn 3500[34]. However, he also mentions that it can be corrected with the correct instrument settings and that minor software adjustments could rectify the problem[34]. In a study by Pastor *et al*[54] on the usefulness of the Sysmex F-800 for canine and feline haematology evaluation, platelet counts also presented some difficulties. In a study evaluating the Coulter AcT for use in domestic animals, problems were encountered with MCV in canine samples and with platelet counts in equine and

feline samples[23]. Feline and bovine white blood cell counts and bovine haemoglobin values also presented difficulties[23].

1.2 THE CELL-DYN 3500[a]

The Cell-Dyn 3500 is a fully automated haematology analyser. It creates a total white cell count (WBC) and a five-part differential leukocyte count, i.e. neutrophils, lymphocytes, monocytes, eosinophils and basophils with additional information on bands, immature granulocytes, blasts and variant lymphocytes. It also measures all the red cell and platelet parameters. In order to measure all these parameters the analyser uses four measurements channels, which are[1]:

a) An impedance channel, for determining the WBC impedance count (WIC)
b) An optical channel for determining the WBC optical count (WOC) and differential cell count
c) An impedance channel for red blood cells and platelets analysis
d) A channel for haemoglobin determination.

The Cell-Dyn has two modes for sample aspiration, an open mode and a closed mode. When using the closed mode the samples are placed in a sample loader, the tube stopper is pierced and blood is aspirated. After the instrument has aspirated the sample, it is diluted, mixed and the parameters are measured in the different channels.

1.2.1 White Blood Cell Measurements

The Cell-Dyn gives three basic WBC measurements, i.e. the WIC, WOC and the reported white blood cell count. With each sample analysis, the WIC and WOC are measured and the values compared. A flag will be displayed if the difference between the two counts exceeds a predetermined value. The WOC is the primary value reported as the white blood cell count. Differences can be due to resistant red cells, nucleated red cells or fragile white blood cells. Nucleated red cells will be included in the total white cell count of the WIC,

while they will not be included in the WOC count. Fragile white cells will cause a false low white cell count in the WIC, as the lytic reagent can also damage them, while they will still be counted in the WOC. Lytic-resistant red cells will cause interference in the WOC, leading to a large percentage of the WOC count located in the stroma region. The comparison of the two methods allows the instrument to identify and report these abnormalities in an attempt to give an accurate white blood cell count.

1.2.1.1 White Cell Impedance Counting (WIC)

For determination of the WIC, a dilution of the sample is made with diluent (L/N 99231-01) and WIC/HGB Lyse (L/N 99431-01). The latter reagent lyses the red cells and strips the cytoplasm from the white blood cells, leaving only the white blood cell nuclei to be counted, using the aperture impedance method. In order to obtain an absolute cell count, the precise volume of blood that passes through the aperture during the count cycle is known. A volumetric metering process is used to ensure that a precise volume of sample is analysed.

Cells that exit the aperture tend to swirl around and can re-enter the sensing zone. To prevent this, and thus the cells from being counted twice, a von Behrens Plate is located in the WIC counting chamber. The WIC is also corrected for coincidence passage loss. Coincidence passage loss is a reduction in the count due to the fact that two or more cells can pass through the aperture simultaneously. This will lead to the generation of a single pulse with high amplitude and increased pulse area, giving the impression that only one large cell has passed through. The coincidence passage loss can be predicted statistically and can be corrected.

1.2.1.2 White Cell Optical Counting (WOC)

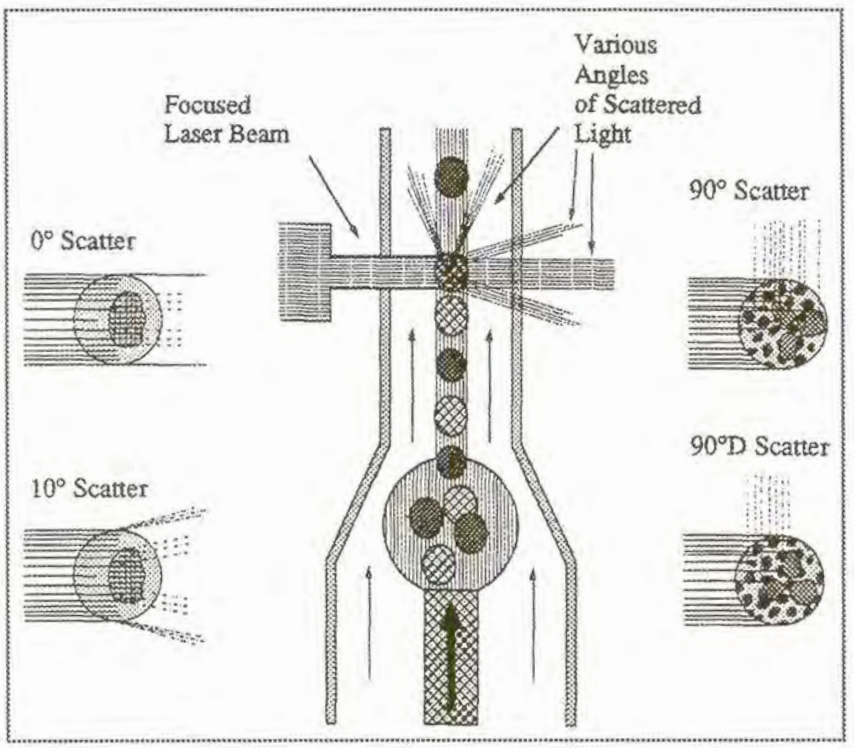
For determination of the WOC, a dilution of the sample is made with the sheath reagent (L/N 99321-01). The cellular integrity of the white cells, in the sheath fluid, is maintained, but the basophils change slightly due to their hygroscopic nature. A measured volume of this dilution is injected into the sheath stream. The cells are aligned in single file as they pass through the WOC flow cell. The WOC flow cell

is an optically clear quartz chamber and the light source is a vertically polarised Helium Neon laser.

The analyzer uses Multi-Angle Polarised Scatter Separation (MAPSS) technology. MAPSS measures the light intensity at four different angles (see Figure 1.1):

- forward angle light scatter (1-3°, referred to as 0°)
- orthogonal light scatter (70-110°, referred to as 90°)
- narrow angle light scatter (7-11°, referred to as 10°) and
- ninety-degree depolarised scatter (70-110°, referred to as 90°D).

Figure 1.1 Multi-Angle Polarised Scatter Separation technology on the Cell-Dyn 3500 (Reproduced with permission from Abbott Diagnostic Division)



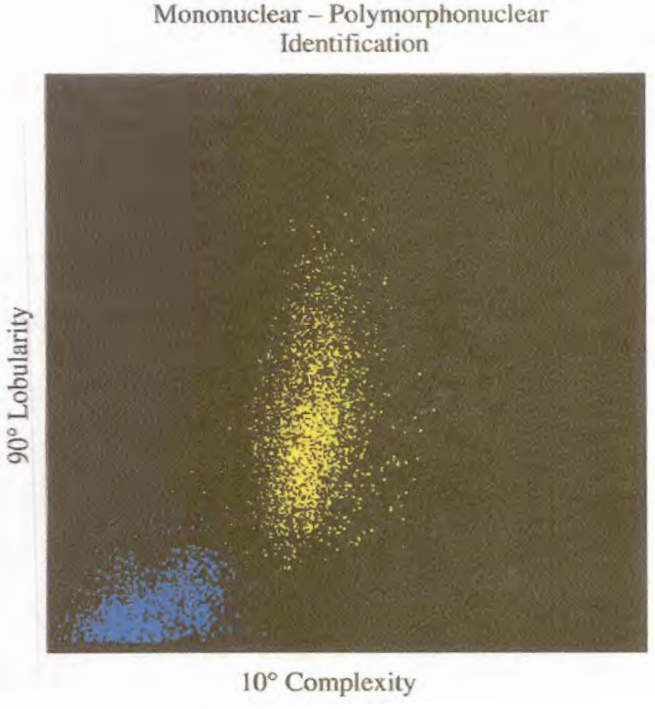
Combinations of these four measurements are used to classify the white cell subpopulations and to give morphological flagging. The number of occurrences above the threshold of the 0° channel is used to determine the white cell optical count. The white blood cell differential count is obtained from all four measurements, and five subpopulations are identified: neutrophils, lymphocytes, monocytes, eosinophils and basophils. The WOC data can be graphically presented as a scatterplot or as histograms.

Mononuclear-Polymorphonuclear Separation

The first step in the differential count of the Cell-Dyn is to classify the cells as mononuclear or polymorphonuclear. The classification is obtained by plotting the 90° (orthogonal) scatter, representing cellular lobularity, on the Y-axis of a

scatterplot against the 10° (forward angle) scatter, representing cellular complexity on the X-axis, see Figure 1.2. The mononuclear cells fall into the lower left part of the scatterplot and above and to their right, the cluster of polymorphonuclear cells is plotted. Once a cell has been classified in either of these two groups, it retains that classification, regardless of it's position in other scatterplots. The instrument determines the best separation between the two populations, using a dynamic threshold.

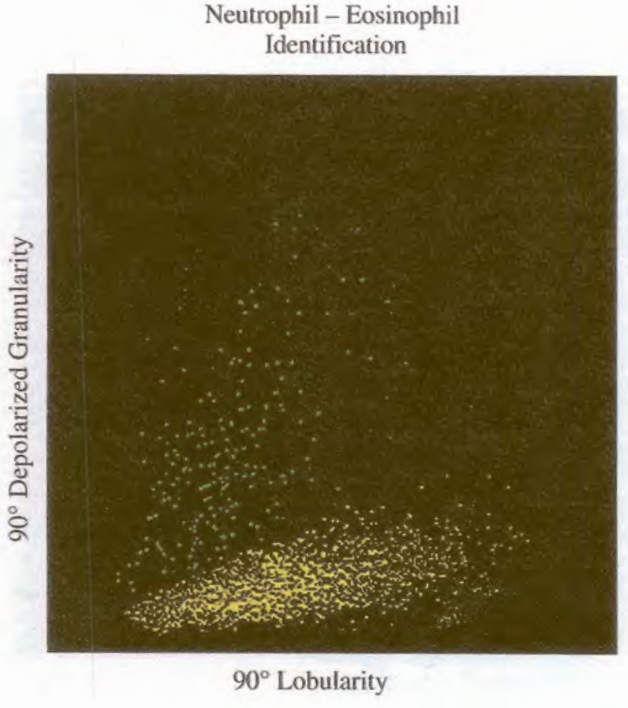
Figure 1.2 Mononuclear-Polymorphonuclear Separation (Reproduced with permission from Abbott Diagnostic Division)



Neutrophil-Eosinophil Separation

In order to distinguish between neutrophils and eosinophils the 90° depolarised light scatter is plotted on the Y-axis against orthogonal light scatter on the X-axis. Eosinophils scatter more 90° depolarised light that other cells due to the unique nature of their granules. Only the polymorphonuclear cells are plotted on this scattergram, the neutrophils are the cells in the lower of the two groups, see Figure 1.3.

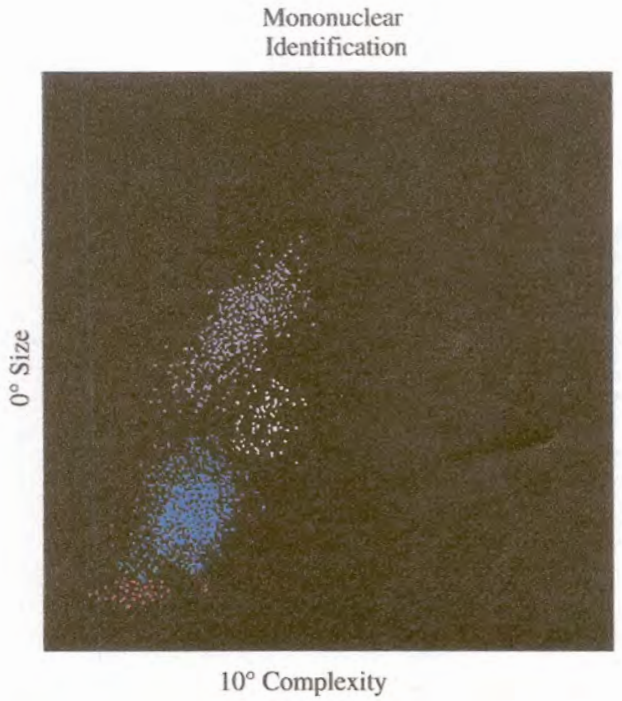
Figure 1.3 Neutrophil-Eosinophil Separation (Reproduced with permission from Abbott Diagnostic Division)



Mononuclear Separation

Lymphocytes, monocytes and basophils fall into this group. The 0° scatter, representing size, is plotted on the Y-axis and the 10° scatter is plotted on the X-axis, see Figure 1.4. The orientation of the neutrophil cluster is also used in the algorithm to help with the classification of the mononuclear cells.

Figure 1.4 Mononuclear Separation (Reproduced with permission from Abbott Diagnostic Division)



Basophils form part of the mononuclear group, because their granules are water soluble in the sheath fluid and in the process the cytoplasm becomes less complex. The lymphocytes are the cells in the lowest, large cluster on the scatterplot. The basophils are present in a small cluster that falls slightly to the right and above the

lymphocytes, whereas the monocytes lie in the big cluster at the top of the scatterplot.

The small cluster below the lymphocytes consists of cells, which are unlikely to be leukocytes. Cell types that can be present in this area are nucleated red blood cells, resistant red blood cells, giant platelets or platelet clumps. The Cell-Dyn uses information from the WIC channel to help with the identification of these particles. Cells in this area will not be included in the white cell count or the differential count.

1.2.2 Data Flagging and Messages

The operator is alerted of any instrument problems and data criteria that may affect the results. The instrument gives the following messages:

a) Instrument messages: Fault and status conditions are given when the instrument detects abnormal conditions during the processing of the sample.

b) Parameter flagging messages:

- Dispersional data flags are given when the numerical values of a given specimen exceed that of the pre-set limits.
- Suspect parameter flags are given when the instrument cannot measure a parameter due to sample abnormality. The flags generated when the white blood cell data cannot be measured are:
  - DLTA: the difference (delta) between the WIC and WOC exceeds the expected, pre-set limit
  - WBC: this flag is also generated when the difference between the WIC and WOC exceeds the limit, but specifically when there is a declining kinetic rate detected for the WOC, or more than 10% of the WOC count was in the stroma region. This usually indicates the presence of red blood cells resistant to the lyse reagent.
  - DIFF (NLMEB): An alert here will indicate a declining kinetic rate for the white cell count, or an abnormal cell population that the instrument cannot reliably classify.

b. 14451839
i. 14756420

- Suspect population flags are generated when the data indicate the presence of an abnormal subpopulation. Flags generated when there are abnormalities detected in the white cell population are:
  - BAND: This flag will be generated when the total number of cells in the band region is more than 12.5% of the total white cell count, or if the ratio of the suspected bands are more that 50% of the total neutrophil count or the neutrophil cluster on the 0° axis appears abnormal.
  - IG: The area where the immature granulocytes (metamyelocytes) are located exceeds 3% of the total white blood cell count.
  - BLAST: The area in the 90°/0° scatterplot where blast transformed cells are usually located exceeds 1% of the total white blood cell count; or the monocyte count is more than 20% of the total white cell count; or the monocyte count is more than 3% of the total white cell count and the monocyte data on the 0° axis is abnormal.
  - VARIANT LYM: The lymphocyte data on the 0°/10° scatterplot exceeds expected criteria.
  - NRBC: This flag is generated when the WIC is greater than the WOC or the area below the WBC threshold on the 0°/10° scatterplot exceeds 5% of the total white blood cell count. Cell types that may be present in this region are nucleated red cells, unlysed red blood cells, giant platelets and platelet clumps.
- Interpretative messages are only printed when the option for it is selected and it is generated when the pre-set numeric limits are exceeded. The white blood cell messages are: leukocytosis; leukopenia; neutrophilia; neutropenia; lymphocytosis; lymphopenia; monocytosis; eosinophilia and basophilia.

The Cell-Dyn 3500 has been evaluated in numerous studies for its use in differential leukocyte counting in human haematology and found to be very satisfactory[19, 25, 31, 52, 62, 77]. It has also been evaluated for its ability to handle and identify pathological samples[12, 36].

1.3 INSTRUMENT EVALUATION

1.3.1 General Instrument Evaluation

There are various levels of instrument evaluation, i.e. evaluation by the manufacturer, evaluation by consumer associations and evaluation by potential users[38, 67]. This paper will deal with the evaluation procedure for use by potential users of the instrument. The different stages of evaluation include a preliminary stage, planning stage, technical assessment and finally an efficiency assessment[9, 10, 37, 38, 67].

During the preliminary stage general information on the instrument must be gathered. This includes the instrument name, manufacturer, marketing company, price, maintenance costs and availability of consumables as well as spares[9, 38, 67]. It should also be determined what space and special requirements, such as water and electrical supply would be required [9, 10, 38, 67].

The planning stage has two legs, i.e. arrangements with the manufacturer and internal resource planning[38]. During the negotiations with the manufacturer, it is essential that a proper assessment of the time required to evaluate the analyzer must be made. An assessment of the reagents and control material must also be made to ensure that sufficient quantities will be available during the evaluation period. Arrangements regarding the service of the analyzer during the time of evaluation must also be made[38].

Internal resources planning revolves around the determination of staff requirements. Sufficient staff should be available for instrument evaluation, special sample collection, keeping daily records, performing the statistical analysis and for reporting on the performance of the instrument[38, 67]. Before technical evaluation starts, it is important that all necessary training of the staff is done[38, 67].

The technical assessment of new analyzers is done by the evaluation of a number of parameters. Evaluation standards for laboratory instruments in general[9] and automated

haematology analyzers in particular[37, 38, 51, 67, 76] have been published. The parameters included in the evaluation are linearity, precision, carry-over, comparability, accuracy and efficiency.

1.3.1.1 Linearity[10, 38, 67]:

The effects of dilution on samples should be assessed for variables whose magnitude should be proportional to dilution. An analytical method should give a linear relation at least over the physiological and commonly encountered pathological range. The linear relation should pass through the origin. Dilutions of a sample are made to give ten concentrations of the sample, i.e. 10%, 20%, 30%, . . . 90% and 100%.

1.3.1.2 Precision:

The precision of an instrument is evaluated by testing a sample on two or more occasions[37, 38, 67]. When a sample is analyzed a number of times, the precision is the degree to which the results vary from the mean of the results[9]. Precision studies should ideally be run over the whole pathological range that is encountered. It is preferable to assay more samples fewer times that to assay fewer samples more times[38, 67], in order to include as wide a range of samples as possible.

1.3.1.3 Carry-over:

The carry-over evaluation assesses the influence of the concentration of the test substance in one sample upon the results obtained for the following sample[9]. Broughton *et al*[9] described a method for the determination of total carry-over assessment. A sample with a high value is used for the first three specimens (h_1, h_2, h_3) and a sample with a low value for the next three (l_1, l_2, l_3). The carry-over between the specimens is then calculated using the following formula[10]:

Carry over = $(l_1 - l_3) \div (h_3 - l_3)$.

1.3.1.4 Comparability and accuracy:

The comparability of an instrument is its ability to produce results that agree satisfactorily with those obtained by accepted routine procedures[67]. The accuracy is defined as the agreement between the best estimate of a quantity and its true value[67]. A true value can only be obtained by a definitive or reference method and the only haematological parameters that can be determined accurately are haemoglobin and packed cell volume[38]. Therefore all other haematological parameters can only be compared to an acceptable routine procedure.

For comparability studies, as many samples as possible should be analyzed to include samples at the extremes of the pathological range. The full pathological range, as expected to be seen in practice, must be included in the analyis[38, 67]. Automated haematology analyzers usually have to be calibrated with a suitable material. This is a big limitation, since this can influence the evaluation. It may be difficult to decide whether an analyzer has been calibrated with a material of which the values were incorrect, or if the analyzer itself is not working correctly[38, 67]. This means that if Analyzer A always give values of 5% greater than Analyzer B, it is acceptable, as the difference could easily have been eliminated by proper calibration. On the other hand, it would not be acceptable if Analyzer A gives a result 10% higher than Analyzer B on some samples and 50% higher or lower on other samples[38, 67].

1.3.1.5 Efficiency:

An efficiency assessment is performed to determine the acceptability of an instrument in a specific working environment. A number of parameters are evaluated to assess this:

- The first factor to take into consideration is the operational time, this includes the throughput, start-up time, shut-down time and the number of samples that have to be repeated[38, 67].

- The next factor to consider is the reliability of the instrument and maintenance necessary. Records of all "down time" must be kept as well as records of the response time of the manufacturer for service[38].
- The format and style of data presentation must be commented on. The ease with which it can be interpreted and the possibility of it being linked to a central laboratory computer should be noted[38].
- Acceptability of the instrument by the staff should be determined. The level of expertise required must be noted[38, 67].
- The costs must be determined and consideration must be given to reagent costs, maintenance costs and costs of consumables. The best way to compare the cost between instruments is to look at the cost per sample evaluated[38, 67].
- The clinical usefulness of the instrument with respect to its screening ability, usefulness as a diagnostic tool and monitoring of patient therapy must be evaluated[38, 67].

1.3.2 Evaluation of Electronic Differential Leukocyte Counters

The difficulties in obtaining an accurate differential white cell count have already been discussed earlier in this chapter. For his reason the National Committee for Clinical Laboratory Standards (NCCLS) has published a standard for leukocyte differential counting that can be used in the evaluation of automated and semi-automated methods for leukocyte differential counting[7, 44, 51].

1.3.2.1 Reference Leukocyte Differential Count[51]

A total of 100 normal and 100 abnormal samples must be evaluated as a basis for the reference values. "Abnormal" is defined as the clinically significant alteration in distribution of mature cell types, or the presence of abnormal or immature cells in clinically significant concentrations.

Whole blood collected by venipucture in tripotassium ethylenediamine tetra-acetate (K_3EDTA) is the required specimen. The sample is rejected if there are any visible signs of clots. Abnormal conditions such as microscopically visible clots, haemolysis and lipaemia should be recorded.

Blood films from each sample must be prepared within 4 hours after blood collection. Samples should not be stored in a refrigerator and should be mixed thoroughly before blood film preparation. Three blood films are prepared on good quality microscope slides. The wedge-pull film technique is used. The films are marked "A", "B" and "spare". The blood films are stained with a Romanowsky stain (containing fixatives) within 1 hour after being made.

The blood films must be examined microscopically using the "battlement" method. Two hundred leukocytes must be counted on each blood film. The cells are classified as segmented neutrophils, band neutrophils, normal lymphocytes, variant lymphocytes, monocytes, eosinophils, basophils and other nucleated cells (excluding nucleated red blood cells). The results are expressed as a percentage of the total number of leukocytes counted. Any nucleated red blood cells are counted and expressed as the number per 100 leukocytes counted.

The examiners must be experienced in examining immature and abnormal cells. They should not perform more than 15 to 25 200-cell differential counts per day.

In 1990 Kohut proposed an abbreviated method for the assessment of electronic leukocyte differential counters[44]. According to his method 20 normal and 20 abnormal blood specimens are evaluated. Four wedge blood films are made and a 400-manual cell count is generated. It is suggested that 4 technologists be used to each scan one of the 4 films in order to generate a 400-manual count. According to Kohut, this reduces the slide-to-slide and technologist-to-technologist variation.