SEED Haematology

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The role of the peripheral blood smear in the modern haematology laboratory

Automated haematology cell counting

The laboratory practice of haematology has evolved tremendously over the past few decades with automated analyser generated complete blood counts (CBC) having fully replaced the original manual individual parameter assay methods. The traditional review of the automated data, and more specifically the white blood cell differential count, of every sample analysed, by means of microscopic examination of the peripheral blood smear has fallen away in most laboratories. The reason for this is the enhanced precision and accuracy of automated cell counting in comparison with the traditional manual counting methods. Moreover, it is also recognised that the superiority of automated differential cell counting is limited to well characterised mature white blood cells, whereas manual microscopy is better for differentiating more immature and abnormal cells where nuances of cytological features are relied upon. In recognition of this, over and above the quantitative data, analysers provide qualitative information in the form of "flags" which alert the operator to the possibility of erroneous results due to interfering variables as well as the presence of abnormal cells.

Based on this, only selective manual peripheral blood smear review on the basis of abnormal quantitative data and flags is required. Whilst the CBC data are accepted without question, it is still commonly a knee-jerk reaction in many institutions to automatically confirm the white blood cell differential by means of microscopic examination of a peripheral blood smear.

The manual peripheral blood smear

Whilst the manual differential count is generally considered to be superior to an analyser generated count for pathological samples, this only holds true under very strict conditions.

The manual differential count is performed by the visual examination of a stained blood smear using a light microscope. A fresh, well-made, peripheral blood smear is crucial for accurate cell morphology assessment. The components contributing towards the final result are fourfold: The quality of the smear, the quality of the stain, the quality of the microscope and the skill and experience of the microscopist.

a) The making of blood smears

The peripheral smear needs to be correctly made. Smears are most commonly made using the "spread" or "wedge" technique. Manual smears are made by placing a drop of blood on one side of a glass slide, and spreading this by rapidly moving a second glass slide or spreader across the first slide at an angle. A well-made peripheral smear is thick (red blood cells are overlapping) at the frosted end and becomes progressively thinner with good separation of cells toward the opposite end. The so-called "zone of morphology", the area of optimal thickness for light microscopic examination, should be at least 2 cm in length. The smear should occupy the central area of the slide and be margin-free at the edges.

Producing a good quality smear requires practice. The blood smear must not be too thin or too thick and the tail of the smear must be smooth. The perfect quality smear is influenced by three factors: speed, angle and drop size.

- The faster the spreader slide is moved, the longer and thinner the smear will be. The slower the slide is moved, the shorter and thicker the slide will be.
- An angle greater than 30° makes the smear thicker; less than 30° the smear is thinner.
- A small drop of blood may be insufficient to prepare a slide of sufficient length; too large a drop may cause the smear to extend beyond the length of the slide.

The viscosity (haematocrit) of the blood, which can be highly variable from patient to patient, will also affect the smear. Blood from a patient with anaemia will have a lower viscosity and the smear will be too thin if the angle is not increased. The opposite holds true for blood from a patient with polycythaemia.

Blood smears that are too thin or too thick present a problem. Extremely thin smears (caused by too small a drop, too slow spreading or too low a spreader angle), may result in red blood cells (RBC) that appear as spherocytes and increased white blood cells (WBC), such as monocytes and neutrophils, in the tails. An incorrect differential will result. In extremely thick smears, the counting area is too small. At least ten low-powered fields where fifty percent of the RBC do not overlap are required for an accurate WBC differential. Blood smears with excessive tails or gritty feathered ends indicate a spreader edge that is rough or dirty, or an accumulation of WBC due to either slow spreading or a very high WBC count.

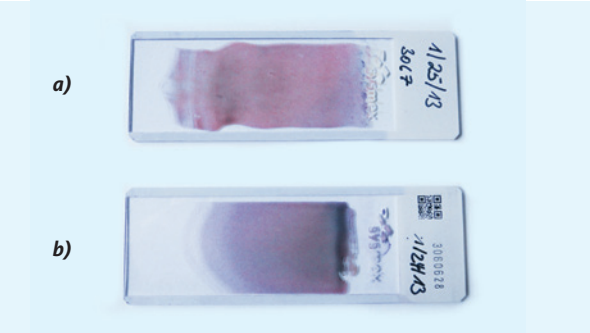

Fig. 1 a) *Poor quality manual peripheral blood smear and* ***b)*** *Good quality automated smear*

b) The staining of the blood smear

The peripheral blood smear needs to be stained so that the cytoplasmic and nuclear details of the various cell types are accentuated. Romanowsky stains or derivations thereof such as Wright, Wright-Giemsa and May-Grünwald-Giemsa are universally used in haematology.

A Romanowsky stain is any stain combination consisting of eosin with methylene blue and/or any of its oxidation products. Such stains produce the typical range of blue, purple and pink colouration of cells when viewed under the microscope (Tab. 1).

	Cellular Component	Colour
Nucleus	Chromatin Nucleoli	Purple Light blue
Cytoplasm	Red Blood Cell Reticulocyte Lymphocyte Monocyte Neutrophil Eosinophil Basophil	Dark pink Grey-blue Blue Grey-blue Pink Pink Blue
Granules	Neutrophil (toxic granulation) Eosinophil Basophil Platelet	Purple (dark blue) Orange-red Purple-black Purple
Inclusions	Malaria parasite	Purple

Tab. 1 *Cellular component colouration in response to Romanowsky staining (Adapted from Dacie and Lewis, Practical Haematology)* [1]

i. How stains work

The mechanism by which certain structural components of a cell stain with a certain dye whereas other similar structures do not, although staining with other dyes, depends on complex differences between dyes, how they interact with each other, as well as the pH of the stain and the cellular micro environment. Acidic structures pick up the basic dye, methylene blue, which as its name implies is blue in colour. In contrast basic or alkaline structures bind acidic dyes, in this case eosin which is pink. An easy way to remember this is to consider the intense pink colour of the eosinophil granules, and the intense purple colour of the basophil granules. The term “eosinophilic” refers to having a likeness or attraction to eosin (the pink dye) and “basophilic” to the basic or blue dye.

Methyl alcohol is used as both a solvent and fixative and forms part of the Romanowsky staining procedure.

ii. Factors giving rise to faulty staining

Many factors can give rise to a poorly stained slide. This may manifest as a slide that is either too dark or too pale, too blue or too pink, granules may appear too dark or are absent, the background may appear blue or there may be stain deposits. Different cellular structures have different affinities for the various dyes – DNA binds rapidly, RNA lags behind and haemoglobin is slowest. Correct timing is therefore critical for a good quality outcome. Having the various dye components in the correct ratio is also critical as they

interact with each other. This aspect of variation is eliminated with the commercially available dye formulations but is a common source of error in home-made stains. As already alluded to, pH of the stain solution is critical. It is generally recommended to be between 6.8 and 7.2. Another common source of error is the overuse of stains, high ambient temperatures resulting in evaporation and contamination from bacteria and other debris.

Automated slide making and staining

Unless the laboratory staff follows strict guidelines in slide making and staining, the probability that poor quality smears will be generated exists. This in turn may give rise to erroneous microscopic interpretation with potential serious consequences for patient care.

In line with the principles of good laboratory practice, standardised slide making and staining procedures will guarantee good quality peripheral blood smears. The best form of standardisation is automation. In most laboratories that do have some form of automation, this is limited to the staining component. Most automated staining systems are so-called "open systems" which allow the use of any stain, including home-made ones, hence only the timing of the staining procedure is controlled. The potential for poor staining due to stain overuse and incorrect composition is not eliminated. Furthermore, the fact that the input into the automated stainer will invariably still be manually made smears, a quality outcome is still not guaranteed.

The other end of the spectrum is a fully automated slide-maker stainer, such as the Sysmex SP-1000i, which is only cost-effective for high throughput laboratories. The good news is that Sysmex, in partnership with RAL Diagnostics, now has a solution to offer the smaller laboratory.

a) The Sysmex HemoSlider

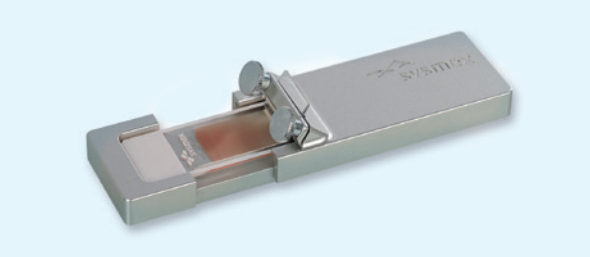

Fig. 2 *The Sysmex HemoSlider*

This is a small, portable, solidly constructed, standardised wedge smear maker. It is easy to use and clean and has replaceable spreader blades. A small drop of blood is placed on the one end of the slide, and the spreader pulled across. The result is a well-made smear of consistent quality with an even spread of cells.

b) The RAL Stainer

Fig. 3 *The RAL Stainer*

The RAL Stainer is an easy to use automated staining unit. The reagents are provided in closed cartridges which have RFID tags which prevent reagent usage beyond expiry as well as over usage, a common problem with other non-closed methods. The staining protocols have been optimised for peripheral blood smears made using the HemoSlider. All sources of error mentioned above can be completely eliminated using the HemoSlider RAL combination. The analyser allows for continuous loading and unloading with the ability to insert an urgent slide at any time. A major advantage is that no maintenance is required and from a safety and environmental perspective, the stains are methanol free.

The stainer has a protocol for bone marrow slide staining as well as for malaria screening. A semi-automated version of this stainer will be available soon. Here an operator will need to move the slide basket from one stain tank to the next, upon the alert from the timer within the analyser. This timer is automatically set in line with the staining protocol selection.

The microscopic review of the peripheral blood smear

Besides a good quality smear and stain, optimal results can only be obtained with a well maintained microscope and a skilled microscopist.

As with all items of laboratory equipment, the microscope requires regular routine maintenance. Hot humid climates are particularly challenging as fungal growth will occur if no precautions are taken. This will damage the optics and render the microscope useless. In contrast, in hot dry climates, the problem is dust.

The microscope needs to be set up correctly to obtain the correct amount of illumination and to eliminate any refractive artefacts which may be mistakenly interpreted as cellular inclusions.

Manual versus automated differential cell counts

For a detailed description of the technical detail of how Sysmex analysers count and differentiate white blood cell sub-populations, please refer to the SEED edition: "Overview of the benefits of switching from a 3-part differential to a 5-part differential haematology analyser". In instances where an automated differential count was obtained, and is subsequently compared with a manual differential count, differences are observed. The reaction to this for most laboratory personnel is to question the accuracy of the automated count.

We need to remind ourselves that the assignment of reference method status to the manual differential count is based on the CLSI guideline of an 800 cell count (2 operators counting 200 cells each on two different smears), and not the routinely practiced single operator single smear 100 cell count.

Furthermore, automated analysers count many more events. For example, a Sysmex analyser will generate a white blood cell differential count from about 10 000 cells as compared with just 100 in the standard manual method. Differences can be quite substantial for various reasons.

a) The unequal distribution of cells in a smear

Unfortunately, even in a perfectly made smear, the distribution of cells is not random. As a rule, neutrophils and monocytes tend to accumulate at the edges of the smear, and lymphocytes in the middle. This problem is accentuated if the smear is too thin or the edge of the spreader is rough. In automated cell counting, cells are in suspension and every single cell in the sample aliquot is counted.

b) Abnormal cell targeting and cell skipping

It is a fact of human nature, that as we scan the slide for a "good area to count", we will hone in on any unusual cells that we see and use that area as our starting point. In this regard, the percentage of abnormal cells may be overestimated. Likewise, the tendency to simply skip any cells that are morphologically difficult to categorise is common practice. The analyser in turn identifies and categorises every cell.

In general, the monocyte count is the one that is most frequently identified as giving discrepant results dependent on counting method. The reasons for this are several fold. Firstly, monocytes tend to accumulate in the tail of the smear which as a rule is not a counting area. Secondly, monocytes can be difficult to distinguish morphologically from activated lymphocytes, and hence are commonly 'skipped' in the 100 cell count. Thirdly, the older the blood is before the smear is made, the greater the disintegration of cells in general and the more difficult the accurate identification of monocytes, in particular, becomes. Eosinophils and basophils, although relatively uncommon, are very distinctive and therefore remain easy to identify. Fourthly, monocytes are a minority cell population so any small differences, introduced by points one to three above, are accentuated. Consequently, automated monocytes as a rule are higher than manual monocyte counts. These differences would be lessened if laboratories were to stick to the CLSI recommendation of the 800 cell count but this is of course not practical, even in the most fastidious of laboratories.

In recognition of the unreliable nature of manual monocyte counting, flow cytometric enumeration using CD45 and CD14 fluorescence-labelled monoclonal antibodies has been proposed as the new reference method for monocyte counting. This has been shown to correlate well with automated counting.[2]

Reference ranges for automated and manual differential cell counts

The way to overcome the apparent differences between automated and manual differential cell counts in general, and monocyte counts in particular, is to adhere to general principles of good laboratory practice and establish reference ranges specific to each method. In this way the values obtained will be interpreted in relation to the appropriate reference and the clinical judgment will not be impacted.

Normal versus pathological cell populations

As described in SEED "Overview of the benefits of switching from a 3-part differential to a 5-part differential haematology analyser", automated analysers alert the operator to the suspected presence of abnormal white blood cells by means of flags. In this instance, a peripheral blood smear should be made and reviewed microscopically.

For completely normal samples though, where no flags are generated, the automated differential count will be more accurate making the manual count obsolete.

Take home message

1. In the era of modern automated technology, there is no need for a manual differential count and peripheral blood smear review of all cases. This should be reserved for those samples identified by the analyser as being suspicious for underlying morphological or cell count abnormalities. This would enable laboratories to utilise the skills and time availability of their staff wisely by focussing on only those smears that truly require their attention.

2. In order to ensure that the microscopic review will provide a report that can be trusted for clinical judgement, the quality of the smear and stain must be optimal. The best way to achieve this is by means of automation of both slide-making and staining. Sysmex has a solution for this means of the Sysmex HemoSlider and RAL Stainer.

3. Differences in manual and automated differential counts, especially for monocytes, will occur. The use of a separate method for specific reference ranges is recommended.

References

[1] ***Dacie and Lewis,*** *Practical Haematology, 9th edition, Edited by SM Lewis, BJ Bain, I Bates, Chapter 4: Preparation and staining methods for blood and bone marrow smears, page 50.*

[2] ***Hübl W, Andert S, Erath A, Lapin A, Bayer PM.*** *(1995): Peripheral blood monocyte counting: towards a new reference method. Eur J Clin Chem Clin Biochem 33: 839 -845.*

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