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New developments in haematology

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Abstract - The blood count dominates the work of the diagnostic haematology laboratory and it is in this area that major advances are being made in automation and in development of new instrumentation. These advances have included the incorporation of processes for measurement of mean platelet diameter and plateletcrit, for display of red-cell and platelet size distributions and for their expression as red cell and platelet distribution width, respectively. Furthermore, the innovations also include differential leucocyte counts which are performed either by continuous flow cytochemistry or leucocyte volume analysis. The clinical usefulness of these parameters will be described and the extent to which the instrument-generated data can be relied on will be considered. In this context, quality control monitoring and calibration by standardised preparations are essential to ensure instrument reliability and inter-instrument harmonisation. These are also important considerations with automated analysers to ensure inter-laboratory comparability of results, especially in the control of oral anticoagulant therapy.

Development of methods based on flow cytometry has resulted in an important change in the technology of haematology, not only in the traditional blood count, but also in other areas. Thus, cell analysis by microfluorimetry is now being used for reticulocyte counting, for identification of monoclonal antibodies and for classification of lymphocyte subsets in leukaemias and other diseases. A further refinement is the use of cell sorters which provide a means for analysis of various blood cells which have been physically separated into discrete homogeneous populations.

INTRODUCTION

The practice of haematology comprises both clinical art and scientific technology. In this lecture, I shall try to assess the impact on both aspects of haematology of some of the technological advances which have taken place. Excluding the development of the microscope on which the art of haematology is based, and the photo-electric colorimeter which we borrowed from clinical chemistry in order to measure haemoglobin concentration, I would like to suggest that there have been two turning points in the practice of laboratory haematology. The first was the invention of the automatic blood cell mixer. Blood is a particulate material in a plasma matrix; reliable analysis of the particulate material is a major haematological concern. Adequate suspension of the particles in a sample to be analysed is of paramount importance. The mechanical mixer began to replace manual "shaking" of the specimens in the early 1950s (1), and this ensured, for the first time, reliable sample homogeneity, and, at the same time, saved time and energy which could be used by the technician for other work.

THE ELECTRONIC CELL COUNTER

The second development was the advent of the electronic cell counter. As early as 1934, Moldavan (2) had described a technique for counting particles in suspension by means of photoelectric detection of scattered light. The potential of this procedure in haematology was not appreciated sufficiently and its development was not promoted until the 1950s when a sudden outburst of productivity saw the development of the first generation of modern blood cell counters. In the past few years these have completely revolutionised the practice of haematology. The counters in general use are based on two principles. In one cells in suspension flow past a light source and the light scattered or reflected gives rise to pulses (3). Their number and size provides the measurement of blood count and blood cell size. The Technicon Hemalog is a prototype of this system (4). A modification of this system uses a laser light beam. The second principle used for measuring cells by means of the pulses which they generate is the electrical impedance method which was invented by

Wallace Coulter (5). At first this was used exclusively in the Coulter Counter but it is now the basis of cell counters made by a number of other manufacturers, although the Coulter Counter remains the eponymic prototype. In the past few years, these counters have been further developed almost beyond recognition, automated and able to perform the most sophisticated procedures. It is, thus, timely to review the changes in haematological practice which these instruments have brought about, and especially to consider the latest developments.

A major achievement has been the ability of the counters to provide measurements with remarkable precision. Thus, haemoglobin concentration (Hb), red blood cell count (RBC) and related parameters can now be measured with a coefficient of variance of 1-2%, and this provides data with a discriminant function which can be used for reliable diagnosis and classification of diseases at a level which was inconceivable a few years ago. It is now possible, for example, to diagnose thalassaemia and to distinguish it from iron deficiency in 99% of cases, using a factor derived from the mean cell volume (MCV), RBC and Hb (6). Similarly, relatively small increases in MCV provide an important means of detecting early stages of megaloblastosis and in identifying other abnormalities of erythropoiesis and liver dysfunction (7). There are limitations in the interpretation of results obtained. As the instruments are essentially comparators, they can be adjusted to give any arbitrary measurements. Thus, unless they are calibrated with reference preparations of known values, their precision will not necessarily be parallelled by an equally high level of accuracy. Instruments based on different principles and even different models of an instrument with minor modifications tend to give different results with some parameters.

Calculation of MCV assumes, not always correctly, homogeneity of the population from which the mean is calculated and a symmetrical distribution curve. It also assumes that the anticoagulant does not affect cell size and shape variably. Differences in the measurement obtained for the MCV occur as a result of different methods for deriving the measurement from the pulses generated. This may either be by dividing the continuous integration of pulse height produced by cells in the sensing zone by the total number of pulses, or by averaging the content of a selected span of adjacent channels in a pulse analyzer. Another factor to account for different measurements on the same sample is the extent to which different diluents affect the size and shape of fresh blood cells and the comparative effect of the diluents on the calibrant. Thus, with the Coulter system, when fresh blood is diluted in Isoton II the cells crenate whereas they retain their natural shape and size when diluted in Isoton III. Red cells change their shape into ellipsoids as they stream through the sensing zone (8, 16) whereas, if a calibrator is used which consists of cells which have been stabilised, these cells will be relatively or absolutely inflexible in the counting system and will thus generate pulses with a different size:shape factor (9).

To harmonise results of measurement with different instruments, there is need for a primary reference preparation. The various manufacturers are working towards this as are the expert panels of the International Committee for Standardization in Haematology (ICSH) and European Community Bureau of Reference. One proposal (10) is to use as primary reference preparations a set of monosized latex particles the size of which would be determined by direct measurement by a metrological procedure. Then by means of a sequential procedure a reference counter would be calibrated by the latices, an intermediate blood standard by the reference counter, and each type of counter used in practice by the intermediate blood standard. Then calibrants for use with similar counters can have a value assigned by the appropriate counter. The intermediate blood standard will be stable for only a short period whereas the latices will be stable for a considerably longer time and will thus serve satisfactorily as a reference; as they are traceable to a primary standard they will fulfil the requirements of certified reference material. It is expected that instruments calibrated with this material will thus be harmonised, with measurements traceably to the primary reference preparations.

Red cell sizing

A notable advance in electronic cell counting systems is the measurement of red cell size distribution. It is now appreciated that MCV alone does not provide adequate information on the actual distribution of cell sizes in a blood specimen and can be misleading when there is a heterogeneous population. Thus, for example, in a nutritional anaemia, iron deficiency with microcytosis may occur at the same time as folate deficiency with macrocytes: the MCV may be normal although the presence of two abnormal populations is clearly demonstrated by discrete measurement of the individual pulses and their presentation as a volume distribution curve. Several published studies have demonstrated the value of these curves in identifying erythrocyte subpopulations (11-14). Characteristic histograms are seen when a patient is responding to haematinic therapy or when there is a population of schistocytes, spherocytes, sickle cells, etc. By means of refined computational processes the data are truncated to exclude inconsistent and aberrant pulses which may give rise to misleading conclusions; theoretical distribution curves are fitted to the edited data and, if the fit is satisfactory, the data are then described by the constants of the theoretical curves. The measurements of clinical interest derived therefrom are then presented on a printout report. There are limitations in interpreting the data. Firstly, it is necessary to ensure that the operative

mode for smoothing curves by editing does not exclude from analysis pulses due to true abnormal populations of blood cells. Conversely, account must be taken of the fact that the character of the pulses may be affected by (a) non-axial flow, with some particles passing obliquely through the aperture, (b) variation in current density, (c) turbulence at the exit side of the aperture with re-entrance of particles to the sensing zone and (d) any flaws in the walls of the aperture. All of these may result in the generation of false pulses (9, 11, 17). In some counters the problem of particle re-entrance has been eliminated by means of either a sheath flow or a sweep flow. In the former the sample is injected into a stream of diluent which forms a laminar sheath surrounding the particles in the sample as they flow through the sensing zone. In the latter a jet of diluent at the exit end of the sensing zone is directed at right angles to the direction of flow of the sample. A hydrodynamic focussing system is another device which enables the stream of particles to pass through a narrow diameter aperture, ensuring that they pass through the aperture axially and separately, thus avoiding distortion of pulses. Fundamental studies have demonstrated the importance of these standardised flow conditions for accurate and rapid measurement (15).
In assessing anaemia, grading the degree of anisocytosis is a time-honoured part of the morphological examination of blood films. This can now be expressed quantitatively as the red cell distribution width (RDW). This is of value in recognising iron deficiency at an early stage, and in distiguishing between iron deficiency and thalassaemia. The usefulness of RDW in other conditions and its potential value in clinical haematology requires an extensive study.

Another measurement which could provide useful information is the distribution of cell haemoglobin concentration, especially in sideroblastic anaemias and in other conditions where anisochromasia is a feature. Experimental studies using a Quantimet Analyser have shown the feasibility of this as a complement to the other cell parameters (18).

Platelet parameters

The platelet count is an important haematological test. It is helpful as a screening procedure in the diagnosis of various blood dyscrasias, essential in the diagnosis and management of thrombocytopenia, and crucial in patients receiving cytotoxic drugs or irradiation. When measured by routine visual haemocytometry the platelet count has a CV of about 30%, and for various reasons, the precision of some commonly used electronic counting procedures is little better. Awareness by manufacturers of these limitations has led to the development of systems which are now able to provide platelet counts with a CV of 8%. These new instruments also provide measurement of plateletcrit, mean platelet volume (MPV) and platelet size distribution (PDW). These parameters are introducing an entirely new dimension to the assessment of platelet function and diagnosis of thrombocytopenia (9, 11, 19, 20).

There have already been several extensive studies on the subject of the inter-relationship of these parameters (24, 11, 20, 25, 26). Normally, there is an inverse non-linear correlation between platelet count and MPV, whilst PDW remains fairly constant. Changes occur as a result of increased destruction and increased production of platelets, disordered megakaryocyte function and primary platelet disorders. Thus, for example, in auto-immune thrombocytopenic purpura (ITP) there is a low platelet count, the MPV is greater than normal and there is marked variation in size, giving rise to a high PDW. With recovery these parameters move towards the normal range, but in some patients in apparent clinical remission, the MPV and PDW remain high despite a normal platelet count. This suggests that, in these cases, platelet destruction continues but there is a compensatory increase in platelet production, rather than a real remission. In ITP platelets are removed randomly from circulation, whereas in hypersplenism large platelets are preferentially sequestered in the spleen and released into circulation only later. Thus, thrombocytopenia occurs in both ITP and hypersplenism but there is a difference in both size of the circulating platelets and their PDW between the two conditions. In hypersplenism the platelets have only a slightly larger MPV than normal and the PDW is relatively normal, by contrast to the high MPV and PDW in ITP.

Normal MPV with increased PDW is characteristic of disordered megakaryocytic function. An increased MPV occurs with the thrombocytopenia of marrow depression induced by cytotoxic drugs. The earliest sign of recovery is a rise in the MPV some days before the platelet count improves (21). The plateletcrit also provides useful information when investigating platelet function as the rate of aggregation depends on the mass of platelets rather than the platelet count (22, 23).

Undoubtedly platelet parameters provide valuable information at an experimental level, but it is not yet clear whether they will become as important as red cell parameters in the clinical context. The observations described above suggest that this will be the case, but further study is necessary to assess the full potential of these measurements.

The problems associated with editing and curve fitting also apply to platelets. Moreover, it is especially important to be aware of the artefacts caused by the effects of EDTA anticoagulants on platelets, changes in platelet size and shape on standing, the possibility of

unrepresentative sampling of the platelet population and the fact that platelets of very small and very large sizes are eliminated from the analysis because the small platelets cannot be distinguished in the counting system from electronic noise at the lowest threshold and the larger ones may be confused with microcytic erythrocytes and other cellular particles at the upper threshold (26). These problems await resolution.

DIFFERENTIAL LEUCOCYTE COUNTS

At the last International Congress on Automation (27), I described the principles of the various automated systems used for the differential leucocyte count. Since then, there have been significant advances in this subject. The instruments are based on three principles: computerised pattern recognition, continuous flow cytochemistry and leucocyte volume analysis.

Computerised pattern recognition was introduced almost 20 years ago by Prewitt and Mendelsohn (28). In principle it uses the method of the traditional differential leucocyte count with Romanowsky stained blood films. A number of instruments have been developed by various manufacturers. Some models have entered the market briefly and have rapidly disappeared. Those which have survived include the Perkin Elmer Diff 4, the Leitz Hematrak, the Omron Analyser from Microx, and most recently the Hitachi Blood Cell Analyzer. There has been surprising hesitation in accepting this type of system in the haematology laboratory. There are several possible reasons for this apparent lack of enthusiasm. Until recently the computerised process of cell identification was relatively slow, not much speedier than manual methods, thus depriving this process of the major advantage of an automated system. Another reason is that the instrument will be no more reliable than the original trainer who has defined the features used by the computer in its decision making; furthermore, reliable performance depends on the consistency of staining between the original training slides, and to achieve this is not easy.

Flow cytochemistry

Continuous flow cytochemistry is the principle used by Technicon in the H6000 system. It is based on the cytochemical reactions of leucocytes. The blood streams into two channels. The first channel is for alkaline peroxidase reaction; 4-chloro-1-naphthol is used as a chromogenic reagent and hydrogen peroxide as substrate at a high pH. In the second channel the heparin granules of basophils are stained with Alcian blue. The stained cells then pass through a flow unit in which they are counted, their size is measured by light scattering and their uptake is measured by light absorption. Unstained cells are classified as small or large. From permutations of size and intensity of staining reaction of the different cells, the instrument can differentiate neutrophils, monocytes, eosinophils, basophils, lymphocytes and another group of cells which are termed "large unstained cells (LUC)". This last group includes some cells of little clinical significance but it also includes blasts. Thus, whilst an occasional cell classified as being in this category is probably of no importance, if they constitute more than 2-3% of the cells in a blood sample, they should be checked by looking at a blood film. If the instrument has too low a level of discrimination, this will mean that a relatively large number of routine specimens will have to be checked, so that the advantage of an automated screening test will be lost. The major advantage of this system is that 10,000 cells are counted in each sample, the procedure taking about one minute. This ensures a high level of precision. It is, however, impossible to judge the accuracy by a cell-by-cell identification based on optical microscopy. Instead, performance can be judged only in terms of clinical reliability, based on the extent to which the instrument is able to distinguish between pathology and normality. In different evaluation reports the false positive rate had been about 12-15% and the false negative rate up to 10% (29). In a recent extensive evaluation (30), Dr. N.K. Shinton and colleagues noted that the differential count obtained by the H6000 was, in general, similar to that from a 200-cell differential count by microscopy; the main discrepancies were in eosinophils and basophils, presumably because insufficient numbers of these cells were seen in a 200-cell manual count to ensure statistically valid data by that method. The instrument is a valuable screening tool, especially if the results are considered not in the light of traditional differential leucocyte counting but in terms of the overall pattern in healthy subjects and the identifiably different patterns in various diseases. The procedure has potential value for subclassifying leukaemias and for identifying anomalous cytochemical reaction (31-32). Thus, for example, some authors have noted low levels of neutrophil peroxidase activity in apparently healthy individuals, a situation which remains to be elucidated (33).

Leucocyte volume analysis

In 1967 Van Dilla working with Fulwyler (34) and Gauthier and Harel (35) separately demonstrated that neutrophils could be differentiated from leucocytes by pulse height analysis of the output from a Coulter Counter, and it was suggested that this method of analysis could be used for carrying out a differential leucocyte count. Subsequently, the practical feasibility of this for clinical purposes was demonstrated by England and his colleagues using a Chanelyzer to resolve lymphocyte and neutrophil peaks in blood samples from normal subjects and random hospital patients on whom a DLC had been requested (36-37).

By analysis of the form of distribution curve it has since been possible to identify several types of cell (38) and to distinguish abnormalities of minor cell classes such as B CLL from other forms of lymphocytic leukaemia (39). The principle has now been incorporated in the Coulter S +II and III in which the percentage and absolute number of lymphocytes are provided with the blood count. A further development in the Coulter S +IV allows further differentiation of the "non-lymphocytes" into neutrophils and monocytes.

It should be appreciated that the measurements do not provide the differential leucocyte counts in the sense of being able to distinguish different cell types by specific characteristics. There is overlapping of the volume of the different cells as presented to the instrument. The volume histogram is manipulated electronically to identify separate peaks and there is no normal leucocyte histogram appearance which can be used as reference, as the major cell populations (lymphocytes and neutrophils) produce a wide variation in proportions, and the histograms are influenced by minor cell populations. In reality, these instruments have created a new technology for assessing leucocytes, and this needs to be assessed to determine whether it provides useful and reliable clinical information. As with the cytochemistry method described earlier, its potential value is in the ability to demonstrate various identifiable patterns which may occur in leuakemia or other diseases, and changes in these patterns with response to treatment or transformation of the leukaemia (11). Nonetheless, the method does provide an alternative to the differential leucocyte count at a screening level, and this should reduce the workload in the diagnostic laboratory. Evaluations of the Coulter S +II and III have shown that the lymphocyte percentage agrees generally with that obtained with the stained blood film. The instrument underestimates the lymphocyte percentage in lymphoproliferative disorders, and when there are significant numbers of reactive lymphocytes present (40, 41) but provided the lack of discrimination is compensated by an adequate flagging for appropriate action when an unusual situation occurs, the purpose of a screening test will have been met.

INSTRUMENT SELECTION

With the ever-increasing numbers of new instruments on the market it becomes a problem of decision for the laboratory director. The choice of counter is to some extent a matter of personal preference but there are certain criteria which will help in the decision. It is necessary to identify and select techniques and instruments appropriate for the work of the laboratory. This should take account of the overall day-to-day workload, the rate and rhythm at which specimens are received by the laboratory, whether the work of the laboratory is restricted to a specialised unit or provides a more general hospital service or is concerned with public health. Account must also be taken of the capital cost and running expenses, the space which is available to accomodate the instrument and the service facilities which may be needed such as water at a particular pressure, appropriate electrical supply, drainage.

All the needs of an individual small laboratory may be served by a simple haemoglobin/WBC counter. In some circumstances, however, the extra data obtained by more complex instruments, even though not of immediate use for patient care, may provide the means for establishing reference ranges and thus improve the predictive value of data obtained in the screening programmes as well as on individual patients. Thus, for example, until relatively recently, the platelet count was considered to be a laborious and unreliable test hardly worth performing except in a few specialised centres. The fact that it can now be carried out rapidly with precision and accuracy has made it an essential test in most laboratories. As I have described above, some of the sophisticated cell counters now also include the differential leucocyte count or an alternative screening test, and this may be an important factor in the choice of an instrument, at least for the larger laboratory. Before making a decision the haematologist should be able to assess critically the information provided by the manufacturer. He should be able to carry out an evaluation of the instrument to ensure that it measures up to his requirements and also to the claims made by the manufacturer. The International Committee for Standardization in Haematology has recently published recommendations for instrument evaluation which provide guidance for this type of study (42).

Quality control

Quality assurance is essential for good laboratory practice, especially so in a laboratory in which automated instruments function with minimal supervision. A traditional method of quality control is to perform duplicate measurements on some of the day's samples and/or repeated measurements on control preparations for plotting a control chart. An alternative method has been developed for continuous monitoring of the performance of a counter, using a weighted analysis of patient data, as described by Bull et al (43). In essence, red cell indices of a population remain stable and will not vary by more than 0.5% from day to day, month to month and year to year provided that the population does not change its essential characteristics. Thus, once the mean value of MCV, MCH and MCHC have been established for a particular centre, any significant deviation from those values in successive batches of tests will suggest instrument drift or bias. The algorithm devised by Dr. Bull ($\bar{x}_B$) is used to reduce the variance by eliminating outliers and by continuously averaging the data. This

procedure has been incorporated into some of the more sophisticated counting systems but it can also be applied readily to other counters by using a programmable calculator or microcomputer (44).

MICROFLUORIMETRY

Microfluorimetry was originally developed as a differential leucocyte count procedure (45). Little data is available for the performance of such a system for this purpose, but the principle is now used extensively for cell analysis in experimental haematology (46, 47). Cells in liquid suspension are stained with a fluorescent dye or combination of dyes and made to flow past a high intensity light source. Fluorescent emissions are detected in photomultiplier tubes which are filtered in order to detect a specific wavelength of fluorescence. Pulses are generated in proportion to the fluorescence of the cells. By stainining with acridine orange which emits a greeen fluorescence when linked to DNA and red fluorescence when linked to RNA, a scatter plot of cellular DNA content against RNA content can be constructed. Another technique allows the instrument to be used with fluorescent-treated antibodies for the identification of monoclonal antibodies and for lymphocyte subset analysis, and there is a rapidly growing literature on these applications (e.g. 48, 49, 50). Other potential applications include identification of platelet antibodies, identification of red cell antibodies, fetal cell counting, reticulocyte counting, etc. Whilst this is essentially a research tool, it is now being used increasingly as a routine diagnostic instrument in at least some specialist laboratories.

A further development is the cell sorter. This is a complex adaptation of microfluorimetry by means of which cells of different subgroups, as identified by their fluorescence, can then be physically separated into homogeneous populations and collected for subsequent analytical studies (51, 52, 53, 54). The potential value of this technique in research is self-evident. But by the natural order of such developments, it may be possible that a clinical version will ultimately be marketed, so that in time to come the haematological profile produced in the diagnostic laboratory will include analytical data of the chemical and immunological content of each cell type present in a blood sample as well as their physical properties.

CONCLUSION

I have confined my talk to developments which relate to blood cells, but automation has also been having an impact on other areas of haemtological analysis. Lack of time prevents me from going into details of the use of automated coagulometers for assay of coagulation factors and for controlling anticoagulant therapy, or automated serology analysers for blood grouping and antibody identification, etc. It is important to appreciate that, in general, these procedures are not merely speeded up versions of manual methods, but that they are often based on different techniques giving reactions which need to be interpreted in their own context and which often provide information other than that obtained by the manual methods. Their value lies not so much in how they help get through the day's routine but in the new areas which they open up.

We have come a long way since Skeggs (55) published a description of an automated analytical method for the measurement of blood urea, since Moldavan (2) described a technique for counting particles in suspension and since Wallace Coulter (5) invented his electrical impedance method. It is, however, well to remember that despite the usefulness and, indeed, essential role of mechanisation and automation in the modern diagnostic laboratory, the haematologist cannot function on automated instruments alone. Despite the automated differential leucocyte count and automated procedures for determining blood cell parameters, microscopy and morphological expertise remain the basis of diagnostic haematology. There will always be an unexpected diagnostic situation which will require human skill for interpretation. Thus, while the instrument system may be becoming increasingly independent of human suprevision, it is well to remember that haematology has always been and remains largely a clinical art. It is vital that we distinguish between having dependable instruments and becoming instrument dependent. Finding the right path is perhaps the greatest problem in automatic analysis in haematology. This requires ever closer collaboration between the manufacturers and the haematologists so that they can share their respective skills for the development of instruments which are both relevant and reliable.

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