Standardmethoden in der Hämatologie

Dieses 1. Seminar mit der Thematik „Referenzmethoden für die Blutzellzählung" veranstaltet von der Deutschen Gesellschaft für Hämatologie und Onkologie in Zusammenarbeit mit der Deutschen Gesellschaft für Klinische Chemie und der Deutschen Gesellschaft für Laboratoriumsmedizin, fand am Samstag, dem 20.11.1993 in Düsseldorf statt.

Wir veröffentlichen in den nächsten drei Ausgaben der Laboratoriums Medizin jeweils Vorträge dieser Veranstaltung.

New developments in establishing reference methods in blood cell counting

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The process of establishing reference methods for the blood count as for all other sets of procedures is continuous. New reference methods must be prepared as new measurements are added to our routine diagnostic armamentarium. Established reference methods must be revised as technology develops and specifications alter. A reference method has been defined by the International Council for Standardization in Haematology (ICSH, 1991) as "a clearly and exactly described technique for a particular determination which, in the opinion of a defined authority, provides sufficiently accurate and precise laboratory data for it to be used to assess the validity of other laboratory methods for this determination. The accuracy of the reference method must be established by comparison with a definitive method, where one exists. A reference method should be traceable to a primary metrological standard and the degree of inaccuracy and imprecision must be stated." The position of the reference method is therefore intermediate in the methodological hierarchy between a definitive method and a selected method. A definitive method is defined as one "which after exhaustive investigation is found to have no known source of inaccuracy or ambiguity, as judged by defined authority. It will, however, have a (known) degree of imprecision which should be stated." A selected method, on the other hand, is "a procedure the reliability of which has been validated by a collaborative study and which is recommended by a defined competent authority as suitable for use in a laboratory analysis, having been selected on the grounds of its accuracy and precision, the intended scope of the test, economy of labour and materials, and ease of operation.

Essentially there are two uses to which a reference method is put. First it is used to assign values to reference materials for the purpose of method calibration and this includes "in house" assignment of values to fresh blood for the purpose of instrument calibration. Secondly reference methods are used to resolve discrepances arising in the comparability phase of instrument or other method evaluations.

A reference method ust never be considered as an isolated and infrequently performed test. Laboratories required to use reference methods must possess the correct equipment including glassware and consumables and must be practised in their use. Every detail of the technique must be assiduously followed. If this is not so then the laboratory may delude itself into thinking that it is performing a reference method, however, in reality the results produced may be highly suspect and misleading conclusions drawn.

Until very recently only two ICSH methods existed for the blood count which were genuine reference methods. The first was for the measurement of the haemoglobin concentration, the most recent version being published in 1987 (NCCLS (1993) have also published a reference method); the second was for the haematocrit measurement (1980). As technology has developed and permitted greater understanding of the problems involved, ICSH activity in the generation of reference methods has accelerated in recent years. At the same time the number of blood count parameters requiring reference methodology has increased as the scope and range of automated blood cell counters has accelerated.

Lack of genuine reference methods was highlighted in the ICSH protocol

The assignment of values to fresh blood used for calibrating automated blood cell counters.
ICSH Cytometry Panel, Clin. Lab. Haemat. 1988, 10, 203-212

"The assignment of values to fresh blood used for calibrating automated blood cell counters" (1988). This document described standardized haematological techniques for assigning values to fresh blood calibrators. The instability of fresh blood restricts its use to short term procedures over a few hours. Such fresh blood calibrators are useful for those laboratories wishing to calibrate their automated blood cell counters themselves without obtaining preserved blood calibrators from other sources. This paper includes specifications for all aspects of the techniques employed including:

- requirements for specimen collection
- glassware specifications including pipettes, capillary tubes, volumetric flasks and counting vials
- methods for direct measurements (reference for haemoglobin and haematocrit; selected for all other methods)
- definition of maximum permissible bias.

Although this paper permitted the use of either K_2 or K_3 EDTA it was recognised that the latter produces shrinkage of cells in the concentration used. This shrinkage results in a 2 % decrease in Packed Cell Volume values. Because of this, ICSH has recommended that for reference methods for the blood count, only K_2 EDTA should be used (ICSH 1993).

Recommendation of the ICSH for EDTA anticoagulation of blood cell counting and sizing.
Amer. J. Clin. Pathol. 1993, 100, 371-372

New recommendations for specimen mixing have recently been published by NCCLS (1993). These specify that, before testing, the specimen container should be well mixed by gentle inversion. The number of inversions of a container, to achieve adequate homogeneity, is dependent on its type and dimensions. Standard evacuated containers, 10–12 × 75 mm, containing 5 ml blood require an air bubble comprising at least 20 % of the tube volume. Non-standard tubes, particularly narrower tubes require more inversions.

Reference methods for erythrocyte and leucocyte counting are now published.

Reference method for the enumeration of erythrocytes and leucocytes.
Clin. lab. Haemat. 1994, 16, 131-138

Both employ a semiautomated single channel aperture impedance instrument operating on a known displaced volume basis without sheathed flow. There presently exists no equivalent light scatter instrument. The method describes instrument specifications in detail, diluent specifications, how to validate thresholds and verify coincidence correction. The instrument must be set up to discriminate erythrocytes and leucocytes. Pulse height analysis is used to verify threshold settings. For erythrocytes it must be established that particles in the range 20–35 fl constitute < 0.5 % of those of volume > 35 fl. Volumes in fl are for erythrocytes and not latex calibration of the pulse height analyser. If this is so, the lower threshold is set at 35 fl and the upper level at infinity. For leucocytes it is necessary to establish, again by pulse height analysis, that particles in the size sample 35–45 fl are < 0.5 % of those of volume > 45 %. If this is so then the lower threshold is set at 45 fl and the upper at infinity. Since coincidence is a function of cell concentration, the adequacy of coincidence correction can be verified using a simple dilution experiment. This method has been described by Lewis et al (1989).

Coincidence correction in red blood cell counting.
Lewis SM, England JM, Kubota F, Phys. Med. Biol. 1989, 34, 1239-1246

A correction for coincidence is made by using regression analysis with analysis of variance to check for non-linearity of the regression, the data points for the analysis being derived from two sets of four secondary dilutions. If the analysis of variance fails to show evidence of non-linearity, the intercept of the regression line represents the coincidence corretected count.

Although none dispute the validity of the original ICSH reference method for the haematocrit (1980), this method is not universally acceptable on the North American continent as a result of restrictions on the use of certain radionuclides. There is therefore need for a revision of the method. ICSH has started work on this. A method based on simple centrifugation is not suitable for reference purposes and thus the revised method will be based on dye dilution using a non-isotope label which does not adhere to erythrocytes. Proof that such is the case must be an integral part of the method. Studies are currently under way to develop such a method which it is hoped will be available in about 2 years' time. An additional advantage will be that, coupled with the reference method for erythrocyte enumeration, these will automatically permit a reference method for the MCV.

ICSH declared its interest in the subject of cell volume distribution as early as 1982 with a paper describing the principles of cell volume distribution analysis.

CSH recommendations for the analysis of red cell, white cell and platelet size distribution curves: General principles.
J. Clin. Pathol. 1982, 35, 1320-1322

This initial publication (ICSH, 1982) provided recommendations for the standardization of cell volume

analysis since the use at that time of many different techniques by researchers and manufacturers alike made it impossible to compare results. Four different issues were addressed:

1. the requirements for fitting a reference distribution
2. the process for fitting a reference distribution
3. the selection of the reference distribution to be fitted
4. the presentation of results.

It is necessary to fit a reference distribution because the cells of interest are usually contaminated by other cells, debris or electronic noise and therefore simple mean measurements and standard deviations would be quite misleading. An iterative process is necessary for fitting a reference distribution. Once one distribution has been fitted, this can be subtracted and attempts made to fit other distributions to the residuum. The analysis only ceases when it has been decided that no residuum of practical significance exists. ICSH recommends selection of a lognormal reference distribution model because this possesses certain major advantages.

1. the lognormal distribution model provides ready comparison of variation in different sizes, e.g. diameter and volume for the same cells.
2. the lognormal distribution is expected to occur in growth situations (for erythrocytes and leucocytes) and in fragmentation (platelet production)
3. cells of negative size are not dedicated.
4. when the VC is < 13 % the lognormal distribution closely approximates the normal distribution.

The following results are presented: the observed size distribution curve, each of one or several curves, the residuum and finally the goodness of fit. Recommendations for the last form the basis of ICSH recommendations (ICSH 1990) a method protocol which was successfully tested by several members of the ICSH Cytometry Panel on a multicentre basis using the hydrodynamically focused Sysmex NE 8000 (McLaren et al, 1993).

ICSH recommendations for the analysis of red cell, white cell and platelet size distribution curves. Methods for fitting a single reference distribution and assessing its goodness of fit.
Clin. Lab. Haemat. 1990, 12, 417-431

The results of this last study provide evidence that laterations in erythrocyte volume distribution can be quantified and in particular that sequential changes can be demonstrated. A similar study has been performed using the Bayer/Technicon H*1, the results of which are currently being evaluated.

The problems surrounding the development of a reference method for platelet counting have taxed haematologists for many years. The problems of basing a platelet count reference method on a chamber counting procedure are well-recognised. In addition, biases are introduced when preparing platelet rich plasma and therefore this method cannot be used as a reference method for platelet counting. It is unacceptable to use other than a whole blood method. There is currently no commercially available blood cell counter which aspirates a known volume of diluted blood through the sensing zone and directly counts platelets in the presence of erythrocytes. For this reason it would only be possible to use an instrument which can determine the ratio of erythrocyte to platelet count. ICSH is currently working on this principle although there are still problems to be solved. Using a sheathed flow instrument which electronically discriminates platelets from other blood cells or debris, the signals from erythrocytes and platelets must be processed, sorted and tallied in separate registers. The erythrocyte count must then be determined using the reference method and the platelet count is subsequently calculated by simple proportion. One such device has been constructed and evaluated (Lewis et al 1990) although it is not available commercially.

Evaluation of a prototype for a reference platelet counter.
Lewis SM, Rowan RM, Kubota F, J. Clin. Pathol. 43, 932-936

The results of this evaluation indication that such an instrument is capable of a high level of linearity and precision, accurate coincidence correction, controlled volume, stability and minimal carry-over. Comparison with established methods was satisfactory and any minor variations noted could equally be due to variability in the traditional method rather than with the instrument under evaluation. This evaluation indicates that the ICSH approach may well be viable but further practical studies needed to be undertaken before such a reference method becomes a reality.

Similarly for a reticulocyte count reference method, the ratio principle may prove successful. ICSH has been examining this issue during the past year and some practical work has commenced.

Finally, there is the differential leucocyte count. The ICSH has kept out of this area since NCCLS (1992) has been expending so much time and effort on the topic. The statistical problems of basophil reference defy solution and remain likely to continue so. This prompts a questioning of the desirability or need to continue to include the basophil count in the routine differential leukocyte count. Problems with the monocyte count reference continue. There is no question of discarding the monocyte component, but is the visual differential count the correct reference procedure? Some workers have questioned the validity of using visual microscopy for the reference method at all (Rowan, 1990; Lebeck et al, 1993).

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Reference methods and materials for counting by instruments using aperture impedance pulse counting (Coulter principle)

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Introduction

Demonstration of valid analytical measurements (VAM) requires reference materials, preferably certified, whose assigned values are traceable to international standards of length or mass.

There are two basic VAM methods. One is to determine a quantity absolutely by means of an accepted procedure in which the errors have been well defined and quantified. In this case the reference materials are those providing traceability of the procedural components, eg. certified balance weights.

The other is to run a simultaneous comparison of the unknown sample with a calibrator. Few devices other than eg. a chemical balance, truly compare two things simultaneously, but for the majority of stable analytical techniques the meaning has been stretched to mean "close together in time". Most haematological instruments are comparitors falling into this category. Although there are now established reference materials and techniques for particle and cell sizing, there are as yet none, other than counting by eye, by which values of number can be certified.

One reference material exists, NIST SRM 1003 glass ballotin [4], with number versus size distribution certified by counting and sizing under the microscope by an internationally accepted body. Only the relative number distribution is given however, the number of beads per gramme of SRM must be computed from the (uncertified) relative density for the glass ballotini. It is also well known that such counts, being very low, have intrinsically poor precision, making them unsuited for the number calibration of count comparison instruments whose results may be several orders of magnitude higher.

Because of this lack of primary references, manufacturers of comparitor instruments usually provide alternative blood based reference materials commercially. Even here, however, apparently only two haematology products claim to be calibrators, ie. to be assayed by traceable techniques, Coulter Scal and Streck Corporation's Cal-Chex.

Latex suspensions are obtainable commercially, with extremely narrow particle size distributions either as single populations (Fig. 1) or mixtures of these, eg. Accubeads from the Fastecs division of Kodak. Mixtures of different bead sizes may cover a very wide overall size range (Fig. 2). Normally, only approxi-