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AN EVALUATION OF RETICULOCYTE COUNTING BY FLOW CYTOMETRY

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A dissertation submitted to the faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirement for the degree of Master of Medicine (in the branch of haematology).

Johannesburg, 1992

ABSTRACT

Since the 1920's, reticulocytes have been known to be a good indicator of bone marrow erythropoiesis. Traditional methods for reticulocyte counting involve staining whole blood with supravital stains and counting 500 - 1000 red cells microscopically (RM). However, there are numerous disadvantages associated with this technique.

Flow cytometric methods of reticulocyte analysis have been introduced recently, which have been shown to be more precise, sensitive and cost-effective than manual microscopic reticulocyte counting. They also allow for the determination of the age of the reticuloyctes, which is known as the reticulocyte maturation index (RMI).

Reticulocytes can be analysed using either a general purpose flow cytometer (RGP) with fluorescent dyes such as thiazole orange, or a dedicated flow cytometric reticulocyte counter (RD), such as the Sysmex R-3000 instrument (TOA Medical Electronics Co, Ltd. Kobe, Japan).

This study compared reticulocyte counting by RGP, RD and RM and assessed the accuracy and precision of the three methods. The RMI was shown to be of additional benefit in the assessment of erythropoiesis.

The RGP was found to be the most accurate technique when looking at all reticulocyte count levels together. The accuracy of RD was not as good. This is most likely related to the fact that the instrument excludes the most highly fluorescent reticulocytes from analysis, as it recognises them as nucleated red

blood cells.

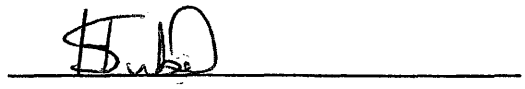
The precision of RD was shown to be excellent, even at very low reticulocyte levels. The precision of RGP was not as good as RD at low reticulocyte percentages, which is most likely related to the problems in setting the negative control using an unstained sample. However, both flow cytometric methods were found to be more precise than RM at all reticulocyte count levels.

The utility of the RMI measurement was demonstrated in a patient with a megaloblastic anaemia on hematinic therapy and in a patient who was treated for a haemolytic anaemia. Problems with flow cytometric methods of reticulocyte analysis in patients with a malaria parasitaemia, chronic lymphocytic leukaemia, chronic myeloid leukaemia and Pappenheimer bodies were demonstrated. No difficulties were observed with reticulocyte analysis in patients with sickle cell disease.

Reticulocyte counting by RGP and RD was found to have numerous advantages over the RM and both flow cytometric methods were shown to be of practical utility in a routine haematology laboratory.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



Helene Linda Subel
24th day of August, 1992

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CLEARANCE CERTIFICATE

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LIST OF ABREVIATIONS

EDTA	Ethylenediamine tetra-acetic acid
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
NCCLS	National Committee for Clinical laboratory standards
CAP	College of American Pathologists
MCV	Mean corpuscular volume
RPI	Reticulocyte production index
FACS	Fluorescence-activated cell sorter
PMT	Photomultiplier tube
A-T	Adenine-thymine
G-C	Guanine-Cytosine
RMI	Reticulocyte maturation index
DiOC[3]	3,3'-dimethyloxacarbocyanine
^{0}C	Degrees celcius
DEQTC	1,3'-diethyl - 4,2'-quinolylthiacyanine
CPD	Citrate-Phosphate-dextrose
FDA	Food and drug administration
LFR	Low fluorescence reticulocytes
MFR	Medium fluorescence reticulocytes
HFR	High fluorescence reticulocytes
UPP	Upper range
CV	Coefficient of variation
mRNA	Messanger ribonucleic acid
PBS	Phosphate buffered saline
FS	Forward scatter
LSS	Log of side scatter
LFL1	Log of fluorescence

CHAPTER 1

RETICULOCYTES

1.1 Recognition of reticulocytes

Reticulocytes were first recognised in 1881 by Ehrlich (cited by Gilmer and Koepke, 1976), due to the appearance of a reticulum in the cells on staining them with methylene blue. The reticulum represents a pre-existing structure, and is not an artifact (Lowenstein, 1959). The reticulocyte count began to be used as an indicator of bone marrow erythropoiesis in the 1920's. The younger the reticulocyte, the more reticulum it contains, as the cell loses reticulum as it matures. Therefore the stage of reticulocyte maturation can be determined by the amount of reticulum in the cell. In 1932, Heilmeyer and Westhäuser described the different stages of maturation of reticulocytes. He divided them into four groups, as well as a group O which represents the normoblasts which have a nucleus and a dense perinuclear reticulum. In group I, the reticulum looks like a dense clump; in group II, it has the appearance of a wreath, in group III, the reticulum has broken up and disintegrated and in group IV there are only a small number of scattered granules or fragments of reticulum present in the cell (Fig. 1) (Friedman, 1984). Every reticulocyte passes through each of these stages in succession as it matures.

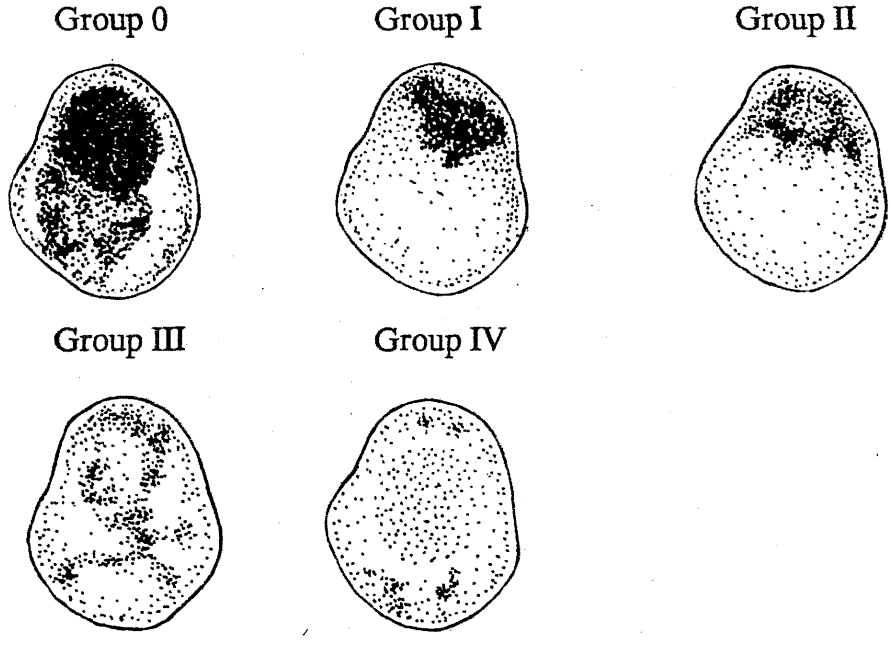


Fig 1. *Heilmeyer Stages of reticulocyte maturation.* (From Friedman, 1984).

Seip (1953) states that the normal percentages of reticulocytes of different maturation stages in the peripheral blood are: 61% group IV, 32% group III, 7% group II and 0,1% group I; the percentages in the bone marrow were reported to be: 0% group IV, 34% group III, 42% group II and 24% group I. Thus, no mature red blood cells are present in the bone marrow under normal conditions. Normally the majority of cells leave the bone marrow as group III reticulocytes (Seip, 1953). These 4 stages of maturation can be separately counted and are valuable in assessing the degree or timing of bone marrow erythropoiesis. However, this is very time consuming, tedious and subjective and so is not used routinely (Thaer and Becker, 1975).

1.2 **Characteristics of reticulocytes**

Reticulocytes are immature red blood cells in their final stages of maturation. They are formed in the bone marrow when the normoblast loses its nucleus. The erythroblast separates into a smaller portion which consists of the nucleus and a thin rim or "corona" of haemoglobinized cytoplasm, and a larger portion which is the reticulocyte. The expelled nucleus is quickly ingested by a nearby macrophage and destroyed (Williams *et al.*, 1990). The immature reticulocyte has mitochondria, ribosomes with RNA, a centriole and golgi body remnants. All of these, but especially the RNA, are precipitated by the supravital dyes to form a reticular structure which gives the cell its name. Different names have been used, including "reticulum, granulofilamentous substance and reticulofilamentous substance" (Koepke and Koepke, 1986). The reticulocyte spends about 2-3 days in the bone marrow and then passes through the wall of the marrow sinusoid. Although different mechanisms have been proposed as to how the traversion through the marrow sinusoid wall occurs, it is still not known exactly how this occurs. Tavassoli and Takahashi (1982) and Lichtman and Santillo (1986) showed that reticulocytes create and pass through

tiny pores in the cytoplasm of the endothelial cells which line the marrow sinuses. The reticulocyte then enters the peripheral blood, where it takes a further 24 to 48 hours before it becomes a mature red blood cell (Hillman and Finch, 1967). Part of this time is spent sequestered in the spleen (Friedman, 1984).

Four factors seem to influence reticulocyte release from the bone marrow: decreasing adhesiveness of reticulocytes as they mature; neural stimulation, possibly via a centre located in the basal part of the brain or via the sympathetic or parasympathetic nervous system; the effect of unidentified humoral substances; the blood oxygen tension (Lowenstein, 1959).

Mel *et al.* (1977) showed that immature reticulocytes are motile, whereas older reticulocytes are non-motile. Reticulocytes also undergo morphologic changes as they mature. Early on, they are multilobular, while as they mature they become cup-shaped (Mel *et al.*, 1977; Mohandas and Groner, 1989).

The membranes of immature reticulocytes are mechanically unstable and rigid when compared to mature red cells (Mel *et al.*, 1977; Mohandas and Groner, 1989). Major structural remodelling of the membrane of the reticulocyte occurs as it matures which allows for improved mechanical stability as well as improved deformability.

Reticulocytes lose their transferrin receptors as well as 90 percent of their insulin receptors and 60 percent of their sodium-postassium pumps as they mature into discoid red blood cells. They also lose membrane phospholipids and so membrane surface area decreases. Also, water is lost from the cells as they mature (Mohandas and Groner, 1989). It has been shown that division of reticulocytes does not occur (Lowenstein, 1959).

While the reticulocyte is maturing, the last third of the cell's haemoglobin is formed (Koepke and Koepke, 1986). Generally it is assumed that the reticulocytes are less dense thatn mature red blood cells, and more dense than normoblasts, based on haemoglobin concentration, so after centrifugation they are found in the top layer of the red cells (Lowenstein, 1959). However, according to Mohandas and Groner (1989), reticulocytes have varying cell densities, based on the changes in hydration of the cells as they mature. As the cell matures the ribosomes and mitochondria disintegrate and are lost from the cell.

The RNA level decreases as the cell matures and a mature red blood cell is formed once the RNA is completely absent from the cell (Friedman, 1984). Reticulocytes have little or no DNA (Holloway and Ripley, 1952; Lowenstein 1959). The reticulocyte count is dependent on numerous factors, including: the rate of red blood cell production; the stage at which reticulocytes are released from the bone marrow; the rate of maturation of reticulocytes in the peripheral blood circulation; the amount of reticulocytes sequestered in the spleen and in pathological states; the rate of reticulocyte and red blood cell destruction by external causes (Cline and Berlin, 1963).

1.3 **Old methods of reticulocyte counting**

Traditionally, reticulocytes have been counted by staining whole blood with supravital dyes, such as brilliant cresyl blue (Cesaris-Demel, 1907 cited by Lowenstein, 1959), new methylene blue (Brecher, 1949) and Azure B (Marshall *et al.*, 1976). These dyes cause the precipitation of the ribosomal RNA in the reticulocytes, forming a deep blue coloured reticulum or dark blue granules, which allows these cells to be recognised (Friedman 1984). According

to Dacie and Lewis (1991), new methylene blue stains the reticulum more deeply and uniformly than brilliant cresyl blue and so gives better results. A few drops of whole blood in EDTA or heparin are mixed with an equal volume of supravital stain, incubated, and then a wedge smear is made on a glass slide (Koepke, 1989). Based on their morphology, these slides are then analysed under the light microscope (Heilmeyer & Westhäuser, 1932), counting at least 500 red cells per analysis. The reticulocyte count is commonly expressed as a percentage i.e. the number of reticulocytes per 100 red blood cells.

1.4 **Disadvantages of manual microscopic reticulocyte counting**

This method of reticulocyte counting is known to be associated with numerous problems, including the time required for analysis, the small number of cells counted, and the variable distribution of red cells (especially with wedge-spread slides). May and Sage (1976) showed that spun slides had a more uniform distribution of red blood cells than wedge spread films. Intraobserver variability also causes problems in interpretation, and Peebles *et al.* (1981) showed that the main cause of inaccuracy is interobserver variability, a significant cause of which is different criteria amongst interpereters as to the morphologic identification of reticulocytes. Differences occured as to whether to call a cell with one or two granules, fine granules or fine filaments of staining, a reticulocyte or not. This is in agreement with the findings of Gilmer and Koepke (1976) who reported that problems occur especially with the most mature reticulocytes, which may have only a single dot of reticulum. This is especially troublesome since as already stated, more than 60% of the reticulocytes in the peripheral blood under normal circumstances are group IV cells. According to the College of American Pathologists (CAP) Survey Program, in order to call a cell a reticulocyte, it must contain two or more clumps or discrete blue granules of reticulum which

are visible without requiring fine focus adjustment (Gilmer and Koepke, 1976). The National Committee for Clinical Laboratory Standards (NCCLS) defines a reticulocyte as any non-nucleated red cell that contains two or more particles of blue-stained material corresponding to ribosomal RNA (Koepke, 1989). Some authorities however, claim that a minimal network of reticulum must still be present, and others only call a cell a reticulocyte if it has 3 dots of reticulum (Koepke and Koepke, 1986).

The above problems occur even when using field restrictors to try to improve the accuracy of cell counting. A commonly used field restrictor is the Miller ocular. When using this device, red cells are counted in a small square and reticulocytes in a large square and then the amount of red cells in the large square is estimated based on the number of red cells counted in the small square. This method assumes that reticulocytes are evenly distributed in dry smears. The technique was reported to be time saving, and to have a standard error less than that of the standard 1000 cell manual count (Brecher and Schneiderman, 1950). However, the overall beneficial effect of such area reducing devices still needs further study (Savage *et al.*, 1985).

The standard error of the proportion of reticulocytes when counted by the manual microscopic method, can be calculated by the formula:

$$Sp = \sqrt{\frac{p\ (1-p)}{m}}$$

Where the p = the proportion of reticulocytes, Sp = standard error, m = number of cells counted overall (Dacie and Lewis, 1991).

Based on this formula, the standard error in reticulocyte counting is inversely proportional to the number of cells counted. Also, the lower the reticulocyte count, the more cells have to be counted to obtain a good estimate of the reticulocyte proportion.. The 95% confidence limits for the reticulocyte percentage can be obtained from published tables, based on the number of red cells analysed. These tables indicate that when few cells are counted ($\pm$ 200), the results are very imprecise and so at least 1000 red cells should be counted (Greenberg and Beck, 1984). Generally, the literature recommends that 1000 red cells are counted per reticulocyte analysis (Savage *et al.*, 1985). Laharrague *et al.* (1990) reported that when at least 5000 red cells are counted, precision is markedly improved.

Peebles *et al.* (1981) also showed that the most significant differences between observers occured at reticulocyte counts of about 1% and higher, but less so when the reticulocyte count was close to zero. Also, greater errors have been found with low reticulocyte counts than high counts (Greenberg and Beck, 1984). Small but significant changes at low reticulocyte percentages are important in detecting reticulocyte responses to blood loss, radiation, chemotherapy and bone marrow transplantation, (Tanke *et al.*, 1983).

Additional complications to counting are siderotic granules (Deiss and Kurth, 1970), Howell-Jolly Bodies, Heinz Bodies and stain debris (Koepke and Koepke, 1986), which all take up the supravital dye and can therefore be erroneously counted as reticulocytes.

According to Friedman (1984), two major factors influence the reliability of the reticulocyte count. The first is physiological, and the second is based on laboratory technique, as has been described above. . The physiological factor is

based on the fact that reticulocyte production has been shown to be diurnal, with higher activity in the morning than in the evening. Other investigations have also reported day to day and seasonal variations in reticulocyte production in normal individuals (Seip, 1953). However, Lowenstein (1959) claimed that these differences are not significant.

Due to all these problems, the clinical usefulness of the reticulocyte count is compromised and so this test, when performed by these techniques, can only be used as a rough indicator of bone marrow erythropoietic activity. Also, it is very difficult to use this method as an accurate reference method with which to compare automated reticulocyte analysers (Peebles *et al.*, 1981).

1.5 **Polychromatophilic Reticulocytes**

When using Romanowsky dyes, no reticulum is formed in the reticulocytes and they appear as diffusely basophilic cells (Lowenstein, 1959). These are called polychromatic, shift or stress cells and correspond to the Heilmeyer group I cells (Perrotta and Finch, 1972) or Heilmeyer group I and II cells (Crouch and Kaplow, 1985) which are displaced from the bone marrow into the peripheral blood circulation. The diameter of these cells is about 25 percent greater than normal mature red blood cells (Perrotta and Finch, 1972) whereas group IV reticulocytes are only 6 percent larger than mature red blood cells (Gilmer and Koepke, 1976). According to Friedman (1984), as the reticulocyte count rises, there is a corresponding increase in the mean corpusular volume (MCV).

When bone marrow erythropoiesis is intensely stimulated by erythropoietin, these shift reticuloctyes are found in the peripheral blood, where they take one to three days longer than the normal circulating reticuloctye to mature.

(Hillman and Finch, 1969). These immature reticulocytes are also found in the peripheral blood in the presence of diseases which affect the bone marrow stroma (Gilmer and Koepke, 1976). In normal individuals, the appearance of polychromatophilic reticulocytes in the peripheral blood is rare, but as haematocrit levels drop, with a corresponding increase in erythropoietin levels, the number of these cells in the peripheral blood increases to a level of about 20% of reticulocytes at a haematocrit of 20-25 (Perrotta and Finch, 1972). These cells are therefore early indicators of bone marrow erythropoietic response to stimulation by erythropoietin (Crouch and Kaplow, 1985).

1.6 **Correction of the reticulocyte count**

The reticulocyte count is used to assess how well the bone marrow is responding to a stimulus for erythropoiesis. The reticulocyte percentage is related to the total red blood cell count, and so it can be falsely elevated in anaemic states. To correct for this, an absolute reticulocyte count can be used i.e. % reticulocytes X red blood cells/mm^3. Alternatively, a reticulocyte index (Friedman, 1984) can be calculated from the formula:

$$\text{Reticulocyte index} = \frac{\text{patient' haematocrit}}{\text{normal haematocrit}} \times \text{reticulocyte \%}$$

Another factor which has to be taken into account is the fact that stress reticulocytes remain in the peripheral blood circulation for longer time periods and so the reticulocyte maturation time is lengthened. As a consequence, the peripheral blood reticulocyte percentage may be falsely elevated and does not provide an accurate index of marrow erythropoiesis. It

has been determined that at a haematocrit of 35 percent, the maturation time would be 1,5 days, at a haematocrit of 25 percent, 2 days and at a haematocrit of 15 percent, 2,5 days (Hillman and Finch, 1967). Theoretically, only the last day of a reticulocyte's lifespan in the peripheral blood should be considered when assessing the bone marrow erythropoietic rate (Koepke, 1989). This is corrected for by the reticulocyte production index (RPI) (Friedman, 1984) which is calculated by the formula:

$$\text{RPI} = \frac{\%\ \text{reticulocytes}}{\text{maturation time}} \times \frac{\text{patient's haematocrit}}{\text{normal haematocrit}}$$

An approximate correction factor can also be used; if shift cells are seen on the blood film, it can be assumed that the maturation time has doubled, and so a value of 2 can be used in the RPI formula (Hillman and Finch, 1969).

In some pathologic states, such as renal failure, even though the patients are anaemic, there is no erythropoietin induced bone marrow stimulation and so no increase in immature reticulocytes in the peripheral blood. Therefore the RPI correction for maturation time is not necessary. So in all anaemic patients, polychromatic reticulocytes must be seen on the Romanowsky film to confirm bone marrow stimulation and so to determine whether the RPI correction would be valid (Hillman and Finch, 1967). The polychromatophilic erythrocyte to reticulocyte ratio has also been shown to be of value in assessing the erythropoietic response of the bone marrow to erythropoietin stimulation in hypoxic conditions (Perotta and Finch, 1972). The RPI has the same normal range as the uncorrected reticulocyte count, as the normal reticulocyte maturation time in the peripheral blood circulation is one day (Koepke and Koepke, 1986).

The normal adult peripheral blood reticulocyte count has been reported as 0,5 to 1,5 percent (Nelson, 1979), 0,5 to 2,5 percent (Dacie and Lewis, 1991) and 1,2 to 2,1 percent for males and 1,6 to 3,3 percent for females (Kaplow and Crouch, 1982). The higher level in females is most likely due to the replacement of menstrual blood losses (Koepke, 1989). At birth, the normal reticulocyte count is higher, with levels of 3-7 percent and 2-6 percent reported during the first 48 hours of life. The count then drops over the next few days to adult levels by the fourteenth day of life (Henry, 1984; Dacie and Lewis, 1991).

Higher reticulocyte counts occur in the presence of increased red blood cell production as occurs in haemolytic anaemias or following the treatment of megaloblastic anaemias (Lowenstein, 1959). However, it has been shown that reticulocytes from anaemic patients have abnormal properties and so caution is necessary when attributing the characteristics of these cells to normal reticulocytes eg. stress reticulocytes have receptors for adhesive proteins which normal reticulocytes have lost by the time they are released from the bone marrow; immature reticulocytes have very rigid, mechanically unstable membranes (Noble *et al.*, 1990); reticulocytes in patients with anaemias due to thalassaemia, chronic uraemia and pernicious anaemia have a delayed rate of disappearance of the reticulum substance, most likely due to metabolic abnormalities of the red cells, and so the rate of reticulocyte maturation is decreased - this factor is disregarded when calculating the RPI (Baldini and Pannacciulli, 1960).

1.7 Clinical utility of the reticulocyte count

The reticulocyte count is still the simplest and most readily available method of assessing bone marrow erythropoiesis. Some authors feel that the raw reticulocyte count is of value in the assessment of certain clinical conditions. The uncorrected reticulocyte count gives an index of red cell lifespan (Crosby, 1981). It can be used to discriminate between anaemias due to bone marrow failure (reticulocyte count decreased) and those due to red cell loss or destruction in which case bone marrow erythropoietic output is increased (reticulocyte count increased) (Koepke, 1989). The reticulocyte count is also useful in monitoring the response of patients with megaloblastic or iron deficiency anaemias to haematinic therapy (Koepke and Koepke, 1986).

When used in its corrected form however, the reticulocyte count is specific for certain conditions. A normal bone marrow should be able to respond to anaemia by increasing the rate of erythropoiesis by up to eight times its normal rate. So the RPI should be at least above two. In the presence of anaemia, the reticulocyte maturation index can be used to distinguish whether it is due to bone marrow failure or ineffective erythropoiesis eg. megaloblastic anaemia (RPI not correspondingly elevated) or red blood cell destruction or removal from the peripheral blood circulation eg. haemolysis, bleeding (RPI elevated ie. RPI elevated at least above 2) or response to therapy eg. megaloblastic anaemia on haematinic therapy (RPI increasing) (Koepke and Koepke, 1986). The RPI is a much better indicator of the different causes of anaemia as it gives a more accurate reflection of bone marrow red blood cell production.

1.8 **Other methods of reticulocyte counting**

The reticulocyte count is an obvious target for automation and recent development are changing the status of the reticulocyte count.

A technique was developed in which a pattern recognition device automatically scanned a new methylene blue-stained blood film. Kaplow and Crouch (1982) reported good precision, reproducibility and correlation with the manual microscopic method using a commercial differential white cell counter (Diff 3^R, Coulter Electronics Inc, Hialeah, FL). The Hematrak automated differential counter (Geometric Data Group, Wayne, PA) also gives good results, although the cells identified as reticulocytes by the instrument need to be reviewed manually (Koepke and Koepke, 1986). Hackney *et al.* (1989) showed that the Hematrak instrument had good precision and accuracy when compared with the manual method and a flow cytometric method using acridine orange, although the precision was not as good as that of the flow cytometric method, most likely due to the fact that fewer cells are counted.

Flow cytometric methods using fluorescent dyes have recently been introduced, and will be discussed in detail in chapter 3.

Immunofluorescence techniques, using antibodies against the transferrin receptor and flow cytometric instruments, have also been tried for reticulocyte counting, but this method only identifies the most immature reticulocytes, as the receptor is lost early on from the red cells as they mature (Seligman *et al.*, 1983). These assays are also more expensive, due to the cost of the reagents (Davis and Bigelow, 1990).

CHAPTER 2

FLOW CYTOMETRY

2.1 Definition

Flow cytometry is the process whereby the measurement of physical and/or chemical characteristics of cells or other biological particles is performed as these cells or particles pass, in single file, through a measuring apparatus in a fluid stream (Shapiro, 1988).

2.2 History of flow cytometry

Shapiro (1988, 1991) has reviewed the historical development of flow cytometry recently. In summary, during World War II the US army evaluated aerosol particle counters, described by Gucker *et al.* (1947) (cited by Shapiro, 1991) for the detection of airborne bacteria and spores. This initiated the development of flow cytometric instruments. Crosland - Taylor, in 1953, used the sheath flow principle and light scattering for the development of a blood cell counter. In 1956, Coulter (cited by Shapiro, 1991) showed that cells in saline raised the impedance of a small orifice as they passed through it in single file. So it was shown that light scattering and electronic volume measurements could be used to detect cells, count them and determine their size. Instruments using this technique became widely accepted in routine haematology laboratories. However, in order to identify cells in mixed populations eg. white blood cell differentials or detection of malignant cells, further measurements were needed . In 1950, Caspersson *et al.* (cited by Shapiro, 1991) showed that the measurement of the absorption of unstained cells using microspectrophotometry could be used to distinguish differences in nucleic acid and protein content of cells with normal and abnormal growth. However, absorption measurements are restricted in their range of applications.

Fluorescent dyes were investigated in an attempt to develop alternatives to the Papanicolaou stain for cervical cytology. In 1950, Friedman (cited by Shapiro, 1991) used fluorescence microscopy and a combination of acid fuchsin, acridine yellow and berberine sulfate to detect uterine cancer; Berberine stains the malignant cell nuclei brighter than normal cell nuclei. In 1951, Mellors and Silver used a scanning microfluorometer to quantitate berberine fluorescence and thus detect cancer cells. In 1955, Von Bertalanffy and Bickis showed that the fluorescent dye, acridine orange, could be used to identify and quantitate tissue RNA content. Acridine orange fluorescence was also used by Hallermann *et al.* (1964) (cited by Shapiro, 1991) to distinguish red blood cells from leucocytes, and granulocytes from mononuclear cells, based on fluorescence intensity. In 1950, Coons and Kaplan conjugated fluorescein isocyanate to antibodies to detect the binding of the antibodies to specimens. Kamentsky (1965) recognised the need for multiparameter analysis of cells. He built the rapid cell spectrophotometer, a computerized optical flow cytometer which measured light scattering at two different wavelengths. In 1967, he added a syringe pump to the instrument to create a fluid cell sorter, to remove selected cells for further analysis.

In 1969, Van Dilla *et al.* used a flow cytometer with an argon ion laser light source and a fluorescent Feulgen stain to measure the DNA content of cells. At the same time, the Herzenbergs at Stanford developed a fluorescence-activated cell sorter (FACS) (Herzenberg *et al.*, 1976).

2.3 Commercial flow cytometric instrumentation

Today, the argon-ion laser is the most commonly used light source for flow cytometers.

In 1973, Becton - Dickinson developed the first commercial FACS instrument (cited by Shaprio, 1991). After that, a series of instruments (FACS Analyzer, FACScan and FACStar) were developed. In 1975 and 1978, Coulter Electronics and Ortho respectively began producing commercial flow cytometers (cited by Shapiro, 1991). Since then, other companies have introduced flow cytometric instruments to the market.

2.4 Multiparameter analysis of cells by flow cytometry

Multiple excitation beam or multistation flow cytometers were developed in the mid-1970's. A number of detectors and filters are used which measure fluorescence at different wavelengths. The detectors are placed at 90^{o} to the laser beam. With these instruments, combinations of dyes with different fluorescence excitation and emission spectra can be used, which allows for multiparameter analysis of cells by detection of forward and 90^{o} light scatter. With specialized high speed electronics, these instruments can detect up to eight different parameters on a single cell. These measurements can be stored as list mode data using a dedicated minicomputer system, for detailed re-analysis at a later stage. So multiple features can be assessed on an individual cell, which is the main advantage of flow cytometry (Shapiro, 1991).

2.5 New Generation flow Cytometers

The early flow cytometers were large in size, complex to use, had high power needs, difficult cooling requirements, and were expensive. These instruments were designed for use in research laboratories.

The new generation flow cytometers are smaller, cheaper, use air-cooled lasers and are more user-friendly. They are therefore better designed for use in the routine laboratory. These instruments also have better optical systems and

microcomputers for instrument control, data acquisition and data analysis. These improvements have helped make flow cytometry an important diagnostic test in the routine laboratory for a wide variety of clinical conditions, with increasing awareness and acceptance by clinicians (Shapiro, 1991).

2.6 Flow cytometric instrumentation

The cells to be studied are first placed in the sample chamber, where mixing and filtering occurs (Fig. 2). They are then transferred under pressure to the flow chamber where they flow in single file down the centre of a stream of sheath fluid. Sheath and sample pressures can be adjusted to give a particular speed of flow of the cells. When the laser or arc lamp light source intersects this stream of flowing cells, measurements are made on each cell separately. This is known as the observation or interrogation point (Shapiro, 1988). Beam-shaping optics are used to focus the laser beam. Most instruments today are multiparameter. In the measurement region, light scattered in the forward direction (forward scatter) is detected as well as light scattered at right angles, which is dependent on the fluorochrome used and the fluorescence intensity (90^{o} light scatter). Forward scatter gives an indication of the diameter or size of the cell, whereas 90^{o} scatter measures either the granularity or internal structure of the cell, or the fluorescence emitted by the cell. Numerous fluorescent probes are available to measure different parameters. The cells absorb the light from the laser and emit different wavelenghts of light depending on the fluorochrome used. These different wavelengths of light are separated using dichroic mirrors and interference and absorption filters (Carter and Meyer, 1990). Photomultiplier tubes (PMT's) are used to detect scatter and fluorescence. These tubes can detect even weak fluorescence. The sensitivity of the PMT can be adjusted on the instrument. The analogue signals from the cells are converted to a digital value and then sent to a computer for analysis, display and storage (Wheeless, 1991) (Fig. 2).

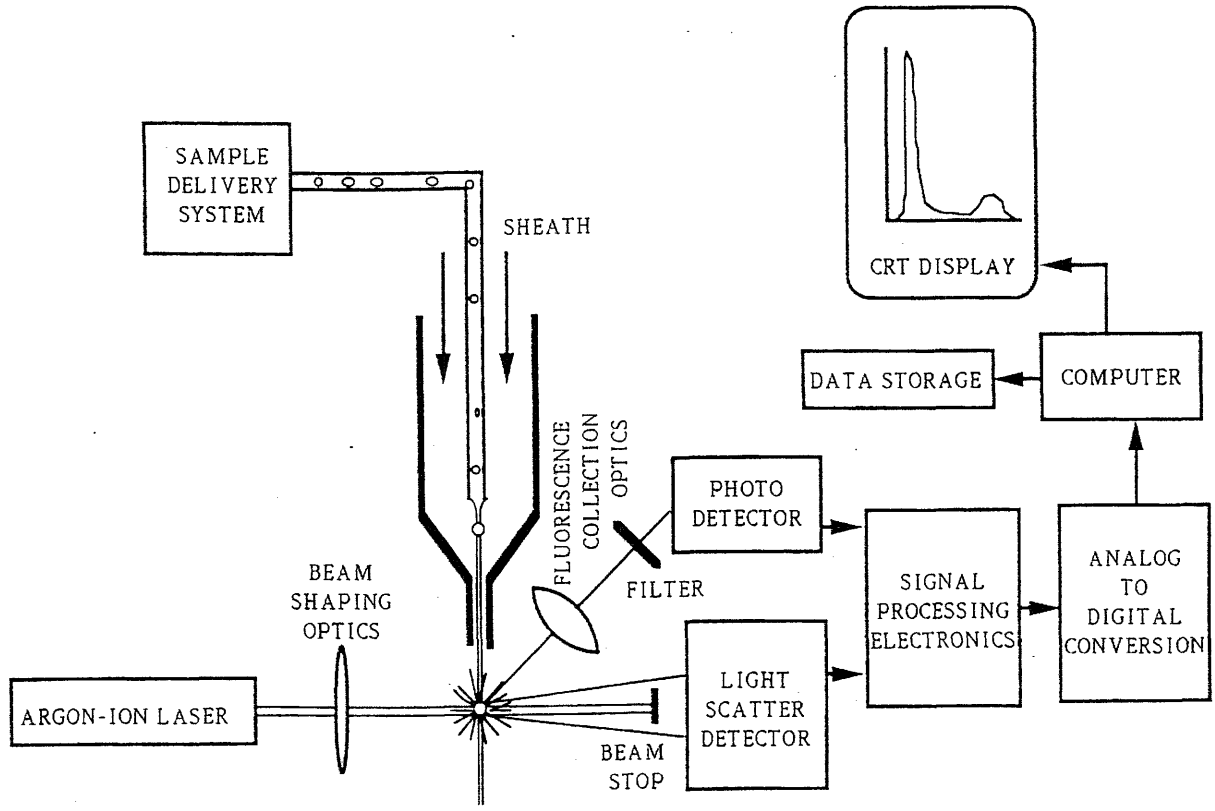


Fig 2. Flow cytometric Instrumentation (from Wheeless 1991).

Logarithmic amplification is useful to measure variables which span a wide range of values, as it gives a better discrimination between points (Wheeless, 1991). Gating is the procedure whereby specific subpopulations of cells are identified for further analysis while the rest of the cells are excluded.

Most instruments also have the ability to sort cells. The stream of cells is broken up into droplets, each containing a single cell. The droplets are charged positive or negative for sorting. They then pass between two deflection plates, and based on their charge, can be deflected out of the main stream. These cells are then collected into different containers (Fig. 2).

2.7 Data analysis

The commonest form of data analysis is by the use of a histogram in which the data is plotted in two dimensions. The units on the horizontal axis are called channels and these represent different signal intensities. One or two parameter histograms can be used. A contour plot can be used to give a good quantitative display of data. Three dimensional plots can also be used (Wheeless, 1991).

2.8 Clinical applications

Flow cytometry has been used mainly for the diagnosis, classification and characterization of leukaemias, lymphomas, and other malignancies. Monoclonal antibodies are used to measure cell surface and intracellular antigens eg. on B- and T-lymphocytes. Also, the DNA content of the cells is measured to determine their ploidy status, which has prognostic significance (Hanson, 1989). Intranuclear antigens can also be detected, using monoclonal antibodies.

The quantification of B- and T- cells is also used to assess patients with immunodeficiencies and organ transplants (Hanson, 1989). In fact, flow cytometry has led to rapid advances in the understanding of the immune system (Giorgi *et al.*, 1992). The use of flow cytometry in the clinical field has been rapidly increased by its application in enumerating the percentages of peripheral blood T-cell subsets in patients with AIDS (Battye and Shortman, 1991).

Reticulocyte determination is another application of flow cytometry and will be discussed in detail in chapter 3.

Antiplatelet and antineutrophil antibodies can also be detected in the serum or in association with cells, using flow cytometry (Hanson, 1989).

Flow cytometry has been used for tissue cross-matching as well, and appears to be more sensitive than the standard lymphocytotoxicity test (Hanson, 1989). Flow cytometry can be used in the blood bank for the quantitation of red blood cell surface antigens or immunoglobulins bound to the surface of red cells, as well as paternity testing (Wong *et al.*, 1989).

Flow cytometry can be used to assess cell cycle status, based on the changes in nuclear DNA content in the different stages of the cell cycle after mitogenic stimulation. DNA synthesis can also be measured by the uptake of bromodeoxyuridine (Hanson, 1989).

Functional assays can also be performed eg. assays of neutrophil activity such as oxidative burst, phagocytosis and opsonization (Hanson, 1989). Functional or physiological parameters such as calcium concentration, pH and membrane potential can be used to assess activation of cells.

Fluorochromes specific for A-T and G-C rich regions of DNA have allowed for the flow cytometric analysis of chromosomes. Specific genetic sequences can also be detected in cells. Analysis of sperm can be performed, based on DNA content. Also, X and Y chromosome carrying sperm can be sorted (Landay, 1989).

Bacteria can be identified using stains for DNA or fluorogenic substrates for enzyme activity. Intracellular parasites can also be identified (Landay, 1989).

Flow cytometry has been used to study the effects of anticancer drugs on the cell cycle. The uptake of drugs into cells as well as the effect that different doses of a particular drug have on the metabolism of cells can also be studied using this technique (Shapiro, 1988).

In summary, flow cytometry has clinical application in a wide variety of fields, including haematology, immunology, histopathology, cytogenetics and blood banking. It can also be applied to microbiology, parasitology, pharmacology and toxicology (Shapiro, 1988).

CHAPTER 3

FLOW CYTOMETRIC METHODS OF RETICULOCYTE ANALYSIS

3.1 Introduction

In 1980, Tanke *et al.*, showed that reticulocytes could be quantitated using flow cytometric methods with suitable fluorescent dyes. Since then, numerous studies have been performed to evaluate the efficacy of reticulocyte counting using either general purpose flow cytometers or dedicated fully automated flow cytometric reticulocyte counters. Alkaline stains are fixed on the RNA of the cells. This gives rise to a complex which is then excited by a laser to emit light, which is detected and analysed by a computer to give a reticulocyte count.

3.2 Fluorescent Dyes

The fluorochromes which have been used for reticulocyte analysis by flow cytometric methods are acridine orange, (Thaer & Becker, 1975; Peebles *et al.*, 1981) pyronin Y (Tanke *et al.*, 1980), auramine - 0, thioflavin T (Sage *et al.*, 1983; Metzger and Charache, 1987) or thiazole orange (Lee *et al.*, 1986).

A number of variables have to be considered when choosing the optimal reagent for flow cytometric reticulocyte analysis, including: the number of reticulocyte tests to be performed, patient population and flow cytometric instrumentation available.

Ideally the dye used should have a simple staining procedure, simple data analysis and the results obtained should be accurate and precise.

The fluorochrome should also have certain physical criteria: an excitation maximum which matches the instrument, a large fluorescent enhancement on binding of the dye to RNA and a high quantum yield.

The dye should also be permeable to the cell membrane so that fixation of the cells during sample preparation can be avoided.

Table 1 summarizes the propoerties of the different dyes which are used for flow cytometric reticulocyte analysis.

Table 1. Fluorescent dyes used for reticulocyte analysis by flow cytometry

Dye	*Stable RMI*	*Quantum Yield*	*Fixation required*	*Fluorescence Enhancement*
Thiazole orange	Yes	Good	No	Good
Acridine orange	No	Low	No	Not good
Thioflavin T	No	Low	No	Good
Pyronin Y	Yes	Low	Yes	Not good
Ethidium Bromide	Yes	-	Yes	-
Propidium Iodide	Yes	-	Yes	-
DiOC [3]	Yes	Low	No	Sensitive to membrane potential changes
Auramine O	Yes	-	No	No

Vander *et al.* (1963) and Seligman *et al.* (1983) used the fluorescent dye acridine orange to count reticulocytes and showed that this method correlated reasonably well with manual reticulocyte counts using brilliant cresyl blue. Thaer and Becker (1975) showed that this dye could be used not only to identify reticulocytes, but also to assess their maturation distribution, using a fluorescence microscope. More recently, this dye has been used to enumerate reticulocytes using flow cytometers. Acridine orange is suitable for use with a laser based flow cytometer with a 488nm line. It binds electrostatically to RNA phosphate groups in the reticulocytes and then stacks

on itself and causes precipitation of nucleic acids. No fixation of cells is required. However, its fluorescence when bound to nucleic acids is complex and depends on the dye concentration, nucleic acid structure, pH and temperature (Davis and Bigelow, 1990). It has a low quantum yield (Koepke, 1989). The total amount of fluorescene which a fluorescent compound emits is dependent on its quantum yield, which is a measure of the number of photons the material emits to the number of photons it absorbs (Shapiro, 1988). The nonspecific binding of the dye to nucleic acids (both RNA and DNA) is also a potential disadvantage. Fluorescence occurs in the red part of the spectrum (Seligman *et al.*, 1983).

Thiaflavin T was proposed in 1983, by Sage *et al.* as a fluorochrome for analysing reticulocytes by flow cytometric methods. Preparation can be performed quickly, no cell fixation is needed and the unbound dye does not need to be removed. However incubation times are critical for both the staining and dilution steps, as the dye leaks out of the reticulocytes after dilution and so this dye may not be practical to use in a busy clinical laboratory (Metzger and Charache, 1987). The dye has a good fluorescence enhancement when it binds to RNA, but a low quantum yield (Lee *et al.*, 1986). Reticulocyte counts can be obtained directly from fluorescence histograms. The dye probably binds to RNA in a similar manner to acridine orange (Metzger and Charache, 1987). This dye can be used with mercury arc based as well as argon-ion laser flow cytometers.

Pyronin Y, ethidium bromide and propidium iodide all require fixation and permeabilization when staining the cells, as the cell membranes of viable cells are impermeable to all of these dyes. One advantage of these dyes is that because the cells are fixed, a stable reticulocyte maturation index (RMI) can be obtained (see later).

The dye, 3,3'-dimethyloxacarbocyanine (DiOC[3]) has a low low quantum yield and therefore gives poor separation between reticulocytes and mature RBC's. Its fluorescence is sensitive to membrane potential changes. It does however produce a stable RMI (see later) (Davis and Bigelow, 1990).

Thiazole orange (1-methyl-4 [(3-methyl-2 (3H)-benzothiazolylidine) methyl] - quinolinium 4-methyl benzene sulfonate) was shown to have properties suitable for flow cytometric reticulocyte analysis (Lee et al, 1986). The dye has affinity for DNA and RNA, intercalates with these nucleic acids in a manner typical of strongly binding intercalating dyes, and is non-precipitating. The dye does not denature RNA when bound to it and therefore causes a stable fluorescence signal, which allows for the determination of the RMI (see later). The staining of DNA as well is a potential disadvantage, but this problem can in most cases be overcome. This dye is excited at 488nm (the optimum wavelength for argon ion lasers). It also has a good quantum yield. Thiazole orange has both nitrogen atoms endocyclic, which causes the "brightness" of the bound dye (which is the product of the quantum yield and extinction coefficient). This factor is partly responsible for the good results which are found with this dye (Lee *et al.*, 1986). In fact, it has a 3000 fold fluorescence enhancement when bound to nucleic acids. It emits fluorescence at 530nm which is in the green part of the spectrum. The dye is membrane permeable, and so no fixation step is required during sample preparation. So the staining procedure and analysis of reticulocytes are both simple when using this dye.

Thiazole orange has a practical disadvantage in that the reticulocyte counts tend to increase artifactually during incubation. This is most likely due to changes in red blood cell permeability which cause non-specific staining of the cells. Lee *et al.* (1986) found that with incubation times of 30 to 90

minutes, there was no significant change in the reticulocyte count. Van Hove *et al.*, 1990 showed that the reticulocyte count remained unchanged over a 2 to 7 hour incubation period, but increased artifactually after 7 hours. The incubation temperature has been found to be less important as the counts did not change with temperatures of $4^{o}C$ to $30^{o}C$ (Van Hove et al, 1990). Ferguson *et al.* (1990), Chin-Yee *et al.* (1991), and Davis *et al.* (1989) reported no significant increase in reticulocyte counts over 48 hours, 72 hours and 96 hours respectively for samples stored at $4^{o}C$ prior to analysis. However, samples stored at $22^{o}C$ showed a significant increase in counts at 24 hours and 48 hours. Bowen *et al.* (1991) had similar results. Another study showed no obvious difference in reticulocyte counts whether blood was stored at $4^{o}C$ or room temperature (Van Hove *et al.* (1990). In contrast, Carter *et al.* (1989) reported a progressive decrease in the reticulocyte count over 24 hours at $21^{o}C$ (room temperature).

Lee *et al.* (1986) showed that increasing the dye concentration shifted the population of red cells to the right and thus overestimated the reticulocyte percentages.

None of the above dyes count as many reticulocytes as new methylene blue without manipulation of the staining procedure or analysis of cells, except for thiazole orange which has been shown to give similar reticulocyte percentages to new methylene blue for counts in the range of 0-35% (Lee *et al.*, 1986).

Van Bockstaele and Peetermans (1989) found a thiazole orange analogue, 1,3'-diethyl - 4,2'-quinolylthiacyanine (DEQTC), which has a chromophoric group similar to thiazole orange, so the physical properties are the same. The only difference is that DEQTC has ethyl instead of methyl substituents and iodide

instead of p-toluene sulphonate. They found a very good correlation when looking at reticulocyte counts with DEQTC and thiazole orange, and so concluded that DEQTC is a valuable alternative for reticulocyte testing.

All the fluorescent dyes have the disadvantage of staining of the sample lines of the flow cytometer, and so the instrument should be cleaned with bleach after each batch of reticulocyte tests have been performed.

Auramine O is another RNA binding fluorochrome, which is used with the Sysmex R-1000 or R-3000 dedicated reticulocyte counters, TOA Medical Electronics Co, Ltd. Kobe, Japan. When used with these fully automated reticulocyte analysers, it also provides a stable RMI (see later). Bowen *et al.* (1991) measured reticulocyte counts with an R1000 instrument and found that specimens could be stored for up to 4 days at $4^{o}C$ in EDTA, with no change in the reticulocyte count. However, when samples were stored at room temperature, there was a small but significant decrease in the reticulocyte count after 24 hours with not much further change thereafter up to 4 days. This suggests that the loss of RNA in reticulocytes does not occur at the same speed *in vitro* as *in vivo*, where reticulocytes are reported to mature within 1 to 2 days (Lowenstein, 1959). They also found that the number of highly fluorescent cells decreased while the number of low fluorescent cells increased with samples stored at room temperature for 4 days. Kojima *et al.* (1989) also found a smaller drop in the reticulocyte count when samples were stored at $4^{o}C$ compared to room temperature especially after 24 hours of storage. These authors also studied the effect of different anticoagulants on the drop in reticulocyte count with specimen storage at room temperature. Using disodium EDTA, heparin and citrate - phosphate - dextrose (CPD), they showed a significant drop in the reticuloycte count after 24 hours although the decrease was less when using CPD compared to the others.

Although flow cytometric reticulocyte analysis can be performed using all of the above dyes, only 2 have been approved by the food and drug administration (FDA) and are commercially marketed. These are auramine O and thiazole orange.

3.3 Evaluation of flow cytometric reticulocyte counting

Very good correlation has been shown when comparing flow cytometric reticulocyte analysis using thiazole orange to manual microscopic reticulocyte counts (Lee *et al.*, 1986; Carter *et al.*, 1989; Nobes and Carter, 1990; Van Hove *et al.* 1990; Chin-Yee *et al.*, 1991). Other flow cytometric methods using dyes such as acridine orange (Hackney *et al.*, 1989; Wearne *et al.*, 1985) and auramine O (Kojima *et al.*, 1989) have also been shown to correlate well with the manual microscopic method. Kojima *et al.* (1989) showed that the results obtained by the Sysmex R-1000 instrument were lower than those obtained by the manual method with low reticulocyte counts. Bowen *et al.* (1991) showed good correlation between reticulocyte counts obtained using thiazole orange and a general purpose flow cytometer and those obtained by the Sysmex R-1000 instrument. Van Hove *et al.* (1990) compared different flow cytometers using the dye thiazole orange and found a good correlation between reticulocyte counts obtained by these intruments.

Some studies have shown a bias in that the reticulocyte counts using flow cytometric methods were slightly higher than with the manual microscopic method (Vaughan et al 1985; Hackney *et al.*, 1989). This may be related to the increased sensitivity of the flow cytometers in detecting low RNA levels (Koepke, 1989).

Flow cytometric reticulocyte analysis using general purpose flow cytometers has been shown to be linear over a reticulocyte count range of 1,8% to 30,1%

(Ferguson *et al.*, 1990) and 0,5 to 8% (Van Hove *et al.*, 1990). The Sysmex R-1000 and R-3000 instruments have been shown to be linear for reticulocyte counts of 0,1% to 15% (Sysmex Operators Manual, R-3000, 1991).

3.4 The Sysmex R-1000 and R-3000 instruments

These instruments are the only fully automated dedicated flow cytometric reticulocyte counters on the market at present. They use a 10mw argon laser light source which has a wavelength of 488nm, and a sheath flow system. (Fig 3).

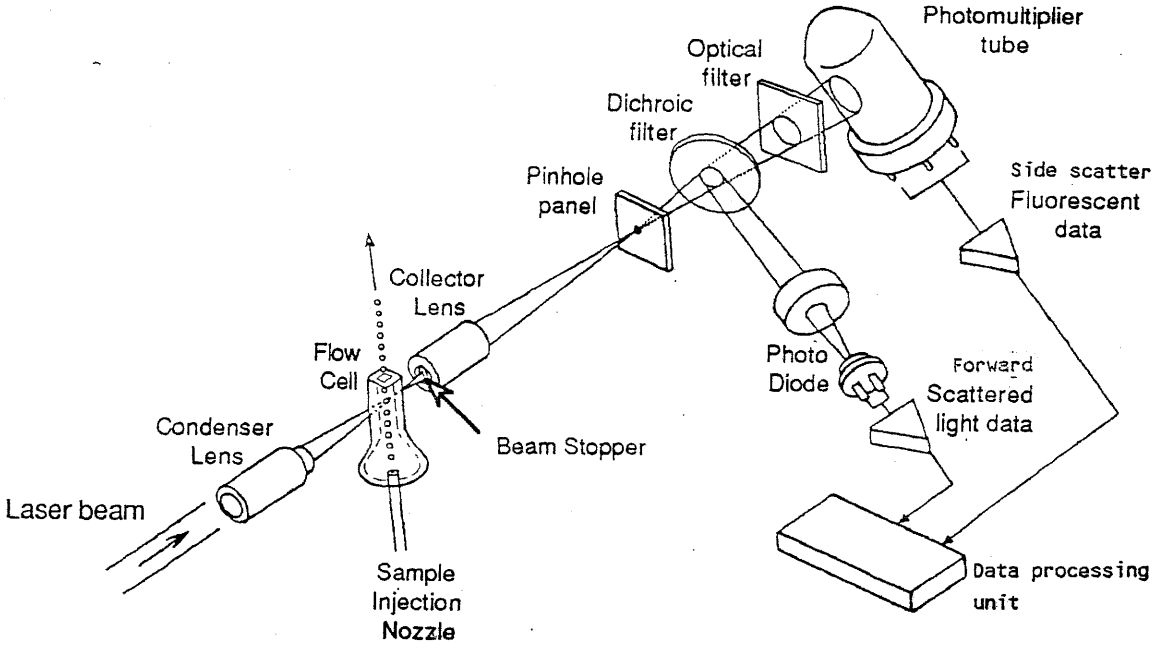


Fig. 3. Optics of the Sysmex Dedicated Reticulocyte Counters (adapted from Sysmex Operators Manual, R-3000, 1991)

The dye used is auramine O, and the light emitted is detected through a green filter. Cell size (forward scatter), and fluorescence intensity which is proportional to RNA content (lateral scatter) are determined and plotted on the Y and X axes of a two-dimensional scattergram respectively. Whole blood in EDTA is well mixed, and then 100μl is sampled. Thirty thousand red cells are analysed per test. Computerized gates are used to exclude platelets and leucocytes, based on their size and fluorescence intensity. The fluorescent reticulocytes and non-fluorescent mature red blood cells are then identified and counted. The channels containing the reticulocytes are divided into 3 which gives an estimate of the percentage of reticulocytes with low fluorescence (LFR), moderate fluorescence (MFR) and high fluorescence (HFR). These correspond to Heilmeyer group IV, II-III and I respectively (Lewis, 1991/92). So the most immature reticulocytes correspond to the HFR's and the most mature reticulocytes to the LFR's. A fourth area, called the UPP (upper range) is not displayed. This area, which is found to the right of the reticuclocyte channels, includes the intensely fluorescent reticulocytes, nucleated red cells and red cells containing Howell-Jolly bodies which are all eliminated from the reticulocyte count. The white blood cells sit in an area even further to the right than the UPP area, and are also excluded from analysis. (Burrichter *et al.*, 1991). The red cell count is also measured and the absolute reticulocyte count calculated (10^9/l). (Sysmex Operators Manual, R-3000, 1991). This instrument has good precision (see later) and counts as low as 0,1 percent are measured (Lewis, 1991/92).

Oyamatsu *et al.* (1989) found a very good correlation between the Sysmex R-1000 instrument and manual microscopic reticulocyte counting when analysing blood samples with Howell-Jolly bodies, Pappenheimer bodies and basophilic stippling. However, when nucleated red cells were present, the correlation was

poor, presumably due to the most immature reticulocytes being counted as nucleated red cells, by the R-1000 instrument, and thus being excluded from analysis. This finding was confirmed by Kojima *et al.* (1989) who found lower reticulocyte percentages being reported in specimens with reticulocyte counts above 10% when using the R-1000 instrument. Cold agglutinated red cells and very large platelets also appear to have an effect on the R-1000 instrument's ability to count reticulocytes. In most of the above cases however, the instrument would show abnormal flags, and so the specimen could be analysed manually. Abnormal reticulocyte results are also obtained in the presence of malarial parasites in the red cells, when using the Sysmex R-1000 instrument (Laharrague *et al.*, 1990; Coulet and Bezou, 1990). Kojima *et al.* (1989) showed no interference with the reticulocyte count in patients with leucocytosis, erythrocytosis, thrombocytosis, hyperbilirubinaemia, hyperglycaemia, lipaemia and partial haemolysis.

3.5 **Reticulocyte identification using general purpose flow cytometers**

The nuclei of white blood cells take up all the fluorescent dyes, except pyronin Y. However, these cells can be separated from the red cells based on their different light scatter patterns and fluorescent intensity which is much higher than that of reticuloyctes.

A histogram representing forward scatter versus side scatter is used to delineate the red cells and exclude the white blood cells and platelets, with bitmapping facilities (Fig. 4).

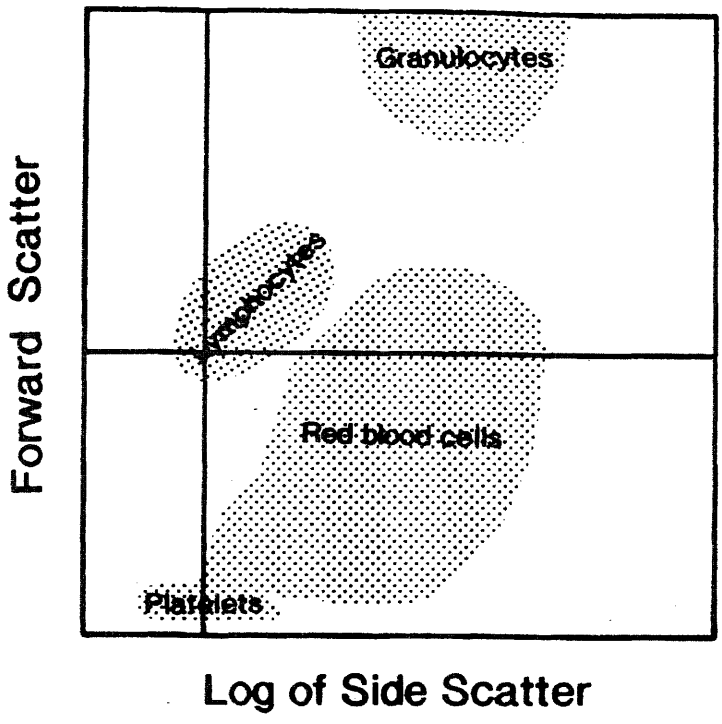


Fig 4. Histogram representing forward scatter versus side scatter

However, small lymphocytes e.g. as in chronic lymphocytic leukaemia (CLL), and large platelets e.g. as in thrombocytopenia, can lie in the same region as the red blood cells and cause problems in interpretation. Very small red cells (MCV < 60 fl) can also cause difficulties in interperetation (Chin-Yee *et al.*, 1991). Furthermore, sickled red cells have been reported to cause problems as they are excluded from the bitmapped red cell population (Ferguson *et al.*, 1990).

Agrawal and Penttil (1992) used a second histogram representing fluorescence 1 versus fluorescence 2, in which leucocytes stained with thiazole orange are seen as a separate population, and can thus be excluded by the use of a bitmap (Fig. 5).

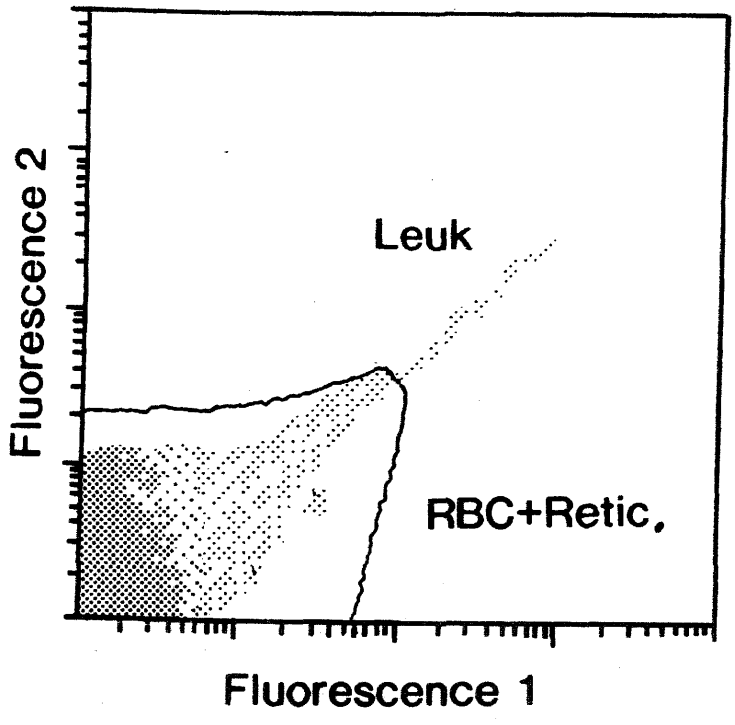


Fig 5. Histogram representing fluorescence 1 versus fluorescence 2 (adapted from Agrawal and Penttil 1992).

The next histogram, representing fluorescence intensity versus number of cells analyses only those cells which have been included in the bitmap on the first, or with Agrawal and Penttil's study, on the first two histograms (Fig. 6).

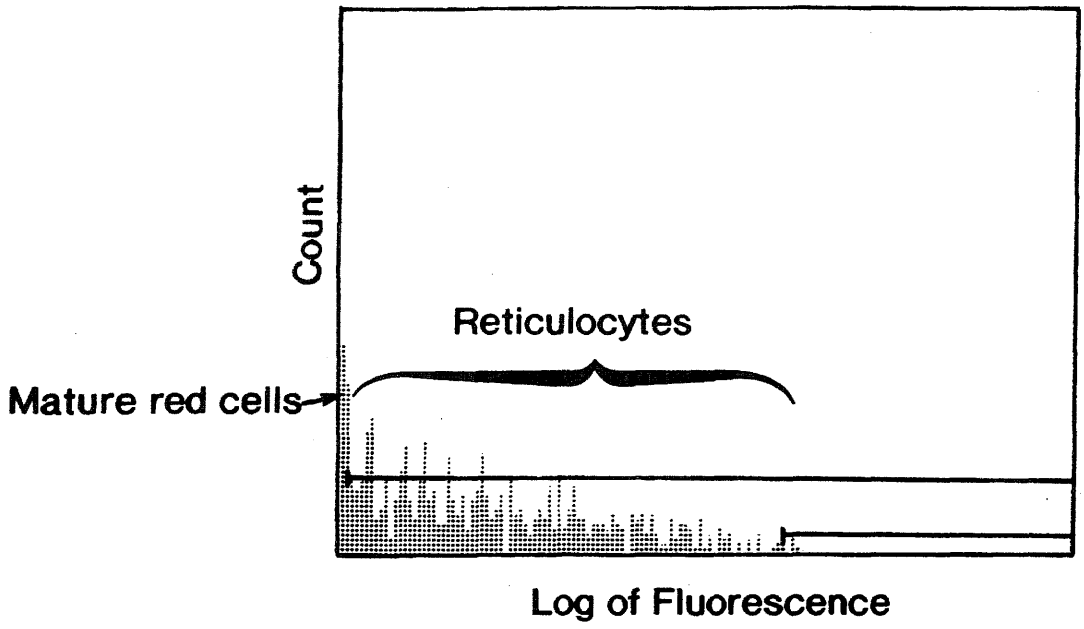


Fig 6. Histogram representing count versus fluorescence

On this histogram representing fluorescence intensity versus number of cells, the major mature red cell peak is seen in the lowest fluorescence channels, and the reticulocytes are seen as a broad population in the higher fluorescence channels, due to their different RNA contents. "Old" reticulocytes (Heilmeyer stages III and IV) are seen in the channels just to the right of the mature red cells, whereas newly released reticulocytes (Heilmeyer stages I and II) are seen in the higher fluorescent channels. The major problem with this technique is the fact that there is no clear point of division between the reticulocytes and mature red cells, but rather a continuous transgression as the cells mature and lose their RNA. The approaches used up until now to separate reticulocytes from mature red cells are either mathematical curve fitting algorithms or the

definition of a reticulocyte based on an unstained control sample. Both of these techniques have been found to give satisfactory results when compared to manual microscopic methods, but further studies are needed to determine where exactly the cut-off point should be (Davis and Bigelow, 1990). Chin-Yee *et al.* (1991) suggested that the cut-off should be at the leading edge of the mature red cell peak which in most cases would give a 0,1% value for autofluorescence. Problems would occur only in the presence of high background autofluorescence, which occurs in patients with reticulocyte counts of >5%, neonates and splenectomized patients. Howell-Jolly Bodies can also take up the fluorescent dye and can thus interfere with reticulocyte analysis, however, they are usually in such low numbers that they are not statistically significant (Davis and Bigelow, 1990). It has been suggested that a blood smear should be examined on all patients who have reticulocyte tests performed, to exclude such inclusions (Chin-Yee *et al.*, 1991).

3.6 **Advantages of flow cytometric methods**

Advantages of the flow cytometric methods of reticulocyte analysis compared to the manual microscopic method have been well reported and include improved precision, sensitivity and cost.

Bowen *et al.* (1991) assessed precision and reported a mean coefficient of variation (CV) of 3,4% with a general purpose flow cytometer, using thiazole orange and a mean CV of 4,3% for the Sysmex R1000 when analysing samples with normal or modestly elevated reticulocyte counts. This is far less than that reported for the manual microscopic method which is in the range of 25-48% (Gilmer and Koepke, 1976) and therefore demonstrates the improved precision of the flow cytometric methods. In another study, Nobes and Carter (1990) using

thiazole orange and a general purpose flow cytometer, reported CV's of 1,42%, 7,3% and 33,3% at reticulocyte counts of 14,3%, 1,8% and 0,1% respectively, again showing improvement when compared to reported CV's for manual microscopic reticulocyte counting. Kojima *et al.* (1989) reported an overall CV of 5,84% using the R1000 instrument. Ferguson *et al.* (1990) also showed a greater precision by flow cytometry than the manual method, with a mean CV of 4,3% by flow cytometry and 22,4% manually. Chin-Yee *et al.* (1991) reported CV's of 2,7-6,3% using thiazole orange and general purpose flow cytometer for reticulocyte analysis

The improved precision with flow cytometric methods is largely due to the fact that much greater numbers of cells are counted with the flow cytometers (20 000 - 50 000) vs 500 - 1000 with the manual microscopic method. Also, the subjectivity of manual microscopic reticulocyte counting is avoided. With the flow cytometric method, thresholds are set to exclude mature red blood cells objectively, based on the analysis of an unstained sample. Therefore, the subjective decision with the manual microscopic method of whether to call a cell a reticulocyte or not, is avoided. (Lee *et al.*, 1986).

The improved sensitivity of the flow cytometric methods is due to the detection of cells with very small quantities of RNA, which would presumably be missed by the manual microscopic method (Van Hove *et al.*, 1990). A 0,2-0,8% positive bias for flow cytometric reticulocyte counts as compared to manual microscopic counts has been reported. Also, higher normal adult ranges have been demonstrated using flow cytometric techniques compared to manual microscopic methods (Davis and Bigelow, 1990).

Ferguson *et al.* (1990) reported an increased speed of reticulocyte analysis using flow cytometric methods especially when samples are run as a batch, which reduces manpower needs. They also claim that although the flow cytometric methods are more expensive, the reduction in time taken for analysis as well as the improved precision and probably accuracy make this technique overall more cost-effective when compared to the manual microscopic method.

3.7 **Normal adult reference values by flow cytometric techniques**

Ferguson *et al.* (1990); Carter *et al.* (1989) and Chin-Yee *et al.* (1991) all showed no significant difference between males and females in the normal adult reference ranges for reticulocytes, using thiazole orange and flow cytometric instrumentation (Table 2).

Table. 2 *Normal adult reticulocyte reference ranges*

Author	Instrument used	Males	Females
Ferguson *et al.* (1990)	General purpose flow cytometer	1,17% - 2,21	1,18% - 2,14
Carter *et al.* (1989)	General purpose flow cytometer	0,26% - 1,22	0,28% - 1,40
Chin-Yee *et al.* (1991)	General purpose flow cytometer	0,9 - 2,9%	1,1 - 3,1%
Tatsumi *et al.* (1989)	Sysmex R-1000	0,81% - 1,33	0,69% - 1,15

Chin-Yee *et al.* (1991) also showed that there is no obvious difference in the normal adult reticulocyte ranges obtained by flow cytometric and manual reticulocyte analysis (1,0 - 3,0% and 0,2 - 2,8% respectively, when combining the results for males and females). Tatsumi *et al.* (1989) also found similar normal adult reference ranges between males and females (Table. 2) using the Sysmex R-1000 instrument.

3.8 **Reticulocyte Maturation Index**

Flow cytometric methods of reticulocyte analysis have the added advantage of being able to estimate the fluorescence intensity of the reticulocyte population. The more immature reticulocytes, corresponding to Heilmeyer stages I and II have the highest fluorescence intensity, whereas the more mature reticulocytes (Heilmeyer Stages III and IV) have a low fluorescence intensity, based on the different RNA content of these cells. Using this feature, one can estimate the overall maturity of the reticulocyte population objectivity. This is known as the reticulocyte maturation index (RMI) (Davis and Bigelow, 1990). The RMI does not always correlate with the reticulocyte percentage and therefore is an additional independent parameter which can be useful clinically (Davis and Bigelow, 1989).

Thiazole orange, as already mentioned, provides a stable reproducible RMI parameter. Samples can be stored at $4^{o}C$ for up to 96 hours, but must be analysed within 2 hours of staining as there is a significant increase in the RMI value after 3 hours of incubation with thiazole orange (Davis and Bigelow, 1989).

General purpose flow cytometers give a "mean channel number" value which is an estimate of the mean fluorescence intensity of the reticulocyte population (Davis and Bigelow, 1989). However the mean channel number has a large variation between and even within individuals. This may be related to the fact that at low reticulocyte counts, very few events are interpreted, and therefore the risk of error is high (Chin-Yee *et al.*, 1991). Davis and Bigelow (1989) found an average CV of 0,8% for the RMI value with reticulocyte counts of 2,1% to 11,4%. Adequate controls need to be developed in order to fully assess the clinical value of the RMI.

Normally, mature reticulocytes with a low RNA content are present in the peripheral blood. However, under conditions of increased effective marrow erythropoiesis, reticulocytes with high RNA contents which are normally present only in the bone marrow, are found in the peripheral blood.

Tanke *et al.* (1983) suggested that pyronin Y could be used to give an assessment of the RMI. They showed the utility of flow cytometric reticulocyte counting post bone marrow transplantation using this dye. They also showed that normal healthy blood donors had a significantly raised RMI value 5-8 hours after blood donation. In 1986, Tanke *et al.* performed a study which demonstrated the value of the RMI in monitoring patients receiving chemotherapy or radiotherapy, using pyronin Y. However, due to variability in staining and the length of the staining procedure when using this dye, it is impractical to use routinely. Reliable RMI measurements are difficult to obtain using thiaflavin T, as the fluorescence intensity is time dependent (Davis *et al.*, 1989). Seligman *et al.* (1983), suggested that acridine orange could be used to estimate the maturity of reticulocytes. However, fluorescent intensity is dependent on the incubation time, and so RMI determination is problematic.

Davis and Bigelow (1989) studied patients post autologous bone marrow transplantation using thiazole orange and a general purpose flow cytometer and found that the RMI values increased as bone marrow function returned. Patients had an increase in the RMI value before, at the same time as, or soon after evidence of myeloid cell recovery. The reticulocyte count, however, did not increase to above 1% until long after neutrophil counts had risen, and is therefore an insensitive indicator of bone marrow engraftment. The RMI value may be especially useful in patients with infection or graft-versus-host disease, in which case the neutrophil counts are not useful in predicting bone marrow recovery (Davis and Bigelow 1989; Davies *et al.* 1992).

The RMI may also be useful in evaluating the response of patients with chronic renal failure to treatment with erythropoietin (Cavill, 1990).

The RMI could also be used to assess the response of patients with nutritional substrate deficiencies to haematinic therapy (Davis and Bigelow, 1990; Cavill, 1990).

The RMI is useful in diagnosing anaemias due to erythroid hypoplasia. These can be either acquired (chemotherapy, toxic reactions) or idiopathic (Davis *et al.*, 1989). Davis and Bigelow (1989) proposed that a true hypoproliferative anaemia can only be diagnosed in the presence of a low reticulocyte count together with a low RMI value.

Iron status has been shown to have an effect on the mean channel fluorescence of the reticulocyte population. In iron deficiency, the RNA level is increased in reticulocytes due to increased amounts of transferrin receptor mRNA, whereas in anaemias of chronic disorders, transferrin receptor mRNA levels are low.

This factor should be taken into account when interpereting the RMI value. This feature may also be useful in assessing the iron status of patients and distinguishing iron deficiency from anaemia of chronic disorders (Wells *et al.* 1992).

Tatsumi and Tsuda (1991) assessed the application of the RMI value, using the Sysmex R-1000 reticulocyte counter. This instrument as already mentioned, divides the reticulocytes into 3 areas, based on low, medium and high fluorescence intensities (LFR, MFR and HFR respectively). These authors found that the changes in these three areas, when looked at separately, were too small to show changes in maturity. They therefore calculated a "maturation index", by the equation:

$$\text{Maturation Index} = \frac{\text{MFR + HFR}}{\text{LFR X 100 (\%)}}.$$

This index was found to be a better diagnostic parameter. It gives an index of the maturity of the reticulocyte population and therefore corresponds to the RMI. The authors showed that this maturation index was useful in patients on chemotherapy and patients with a haemolytic crisis.

Kojima *et al.* (1989) showed an increase in reticulocytes with low fluorescence in patients post- chemotherapy with hypofunctioning bone marrows, and an increase in highly fluorescent reticulocytes in patients with a haemolytic anaemia, using the Sysmex R-1000 instrument. Davies *et al.* (1992) found the Sysmex R-1000 instrument to be useful in evaluating erythropoietic recovery post-bone marrow transplantation, based on the HFR measurement which was found to be an early and sensitive indicator. They also found the absolute reticulocyte count to be of value in predicting bone marrow engraftment.

Kakuya *et al.* (1991) showed the value of the HFR measurement in predicting plasma erythropoietin levels in neonatal anaemia, using the Sysmex R-1000 instrument.

Bowen *et al.* (1991) compared the mean fluorescence intensity of the red cell population using thiazole orange and a general purpose flow cytometer, to the HFR value using the R-1000 instrument, and found a significant, although fairly weak, correlation ($r = 0{,}430$; $p < 0{,}01$).

Nagao *et al.* (1990), using the R-1000 instrument showed that blood samples with a high HFR value also had many Heilmeyer Group I and II reticulocytes by the manual microscopic method using new methylene blue.

In summary flow cytometric reticulocyte analysis is valuable for oncology, nephrology and in fact all clinical disciplines based on the better accuracy and precision of counting as well as the added advantage of the RMI estimation.

However, before the RMI can be accepted as a recognized haematologic parameter with clinical utility, standardized units and normal ranges need to be defined.

CHAPTER 4

AIMS OF STUDY

4. Aims of Study

The purpose of this study was to compare reticulocyte counting by manual microscopic methods (RM), a general purpose flow cytometer (RGP) and a dedicated automated flow cytometric reticulocyte counter (RD). Previous studies have compared RGP with RM, RD with RM and RGP with RD (see chapter 3). However, no studies have been reported comparing the three methods simultaneously.

In particular, specific aims were to evaluate:-

i) the accuracy of reticulocyte counting by the three different methods, which has not been specifically addressed in previous studies.

ii) the precision of each method.

iii) qualitative reticulocyte parameters; specifically the mean channel number (MCN) and high fluorescence reticulocyte (HFR) measurements by RGP and RD respectively were compared, to see if there was a significant correlation between these two values.

The practicalities and advantages of using flow cytometric methods of reticulocyte analysis in a routine haematology laboratory were addressed as well.

CHAPTER 5

MATERIALS AND METHODS

5.1 Specimens

Specimens of whole blood were collected into EDTA tubes as venous blood or by fingerprick. Random blood samples which had been sent to the laboratory for reticulocyte testing were used. Examples of interesting cases which demonstrated certain principles of the different test methods were also assessed. The study was performed during the period from January 1992 to August 1992. Samples were analysed within 24 hours of blood collection, with specimens being stored at $4^{o}C$ prior to analysis, based on the results of previous studies. (see chapter 3).

5.2 Manual Reticulocyte Counting (RM)

Blood samples were stained with new methylene blue, at a 1:1 dilution, vortexed, incubated at $37^{o}C$ for 20 minutes, and then spread onto glass slides and read microscopically using a light microscope and an oil-immersion lens. 500 or 1000 cells were counted by one or two observers respectively for each analysis.

5.3 General purpose flow cytometer (RGP)

Samples were analysed on an Epics Profile II flow cytometer (Coulter Electronics, Hialeah, FL.), which has an argon ion laser operating at 488 nm at a power of 15W. The instrument was calibrated daily using Coulter immunocheck fluorescent latex beads. The dye used was thiazole orange which was prepared by adding 1mg of dye to 1ml of methanol and then this was stored in the dark at $-20^{o}C$ for up to five months. Two microliters of whole blood was added to 2

microliters of thiazole orange reagent in 2ml of phosphate buffered saline (PBS) at a pH of 7,2 - 7,4 and gently vortexed for a few seconds. The samples were incubated at room temperature for 60 minutes in the dark, resuspended and then analysed. A dilution of thiazole orange in phosphate buffered saline of 1:10000 was used for all analyses as this concentration was shown to correlate with the manual microscopic reticulocyte counts and the dedicated flow cytometric reticulocyte counter (see later). The flow cytometer was set to count 20 000 cells per analysis after the red cell population was bitmapped on a forward scatter versus log of side scatter (90^{o} angle light scatter) histogram. On this histogram, the red cells represent the majority of cells seen as very low numbers of white cells are present compared to red cells. The white cells and platelets are also represented in a different position to the red cells on this histogram, and thus can be excluded by the bitmapping facilities (see Fig. 4).

A second histogram of forward scatter versus log of fluorescence was utilized to further eliminate the white cells with the use of bitmapping facilities. The white cells have a higher fluorescence intensity than the reticulocytes as the dye is taken up by their nuclei. They therefore lie to the right of the reticulocytes on this histogram and so can be excluded (Fig. 7). No previous studies have used this histogram in this context.

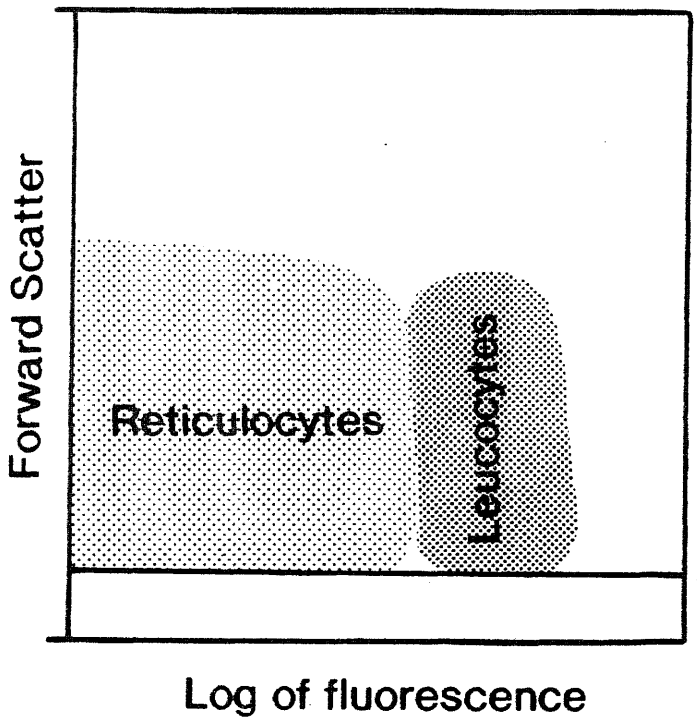


Fig. 7. Histogram representing forward scatter versus log of fluorescence

The third histogram used was that representing number of cells (count) versus log of fluorescence (Fig. 8). A logarithmic frequency scale was used to improve the discrimination between the red cell peak and the reticulocyte distribution. Only the cells included in the bitmaps on the first two histograms are represented here. The mature red cell peak occurs in the lowest fluorescence channels and the reticulocytes as a broad population in the higher fluorescence channels, with fluorescence intensity being proportional to the RNA content of the cells (Fig. 8B). Negative controls, consisting of PBS and whole blood alone were prepared and analysed under identical conditions with each test. Gates were set to include not more than 0,3% background fluorescence in the area in which the reticulocytes would be counted (Fig. 8A). The count obtained in the reticulocyte area (background fluorescence) on the control sample was subtracted from the reticulocyte count obtained on the test sample.

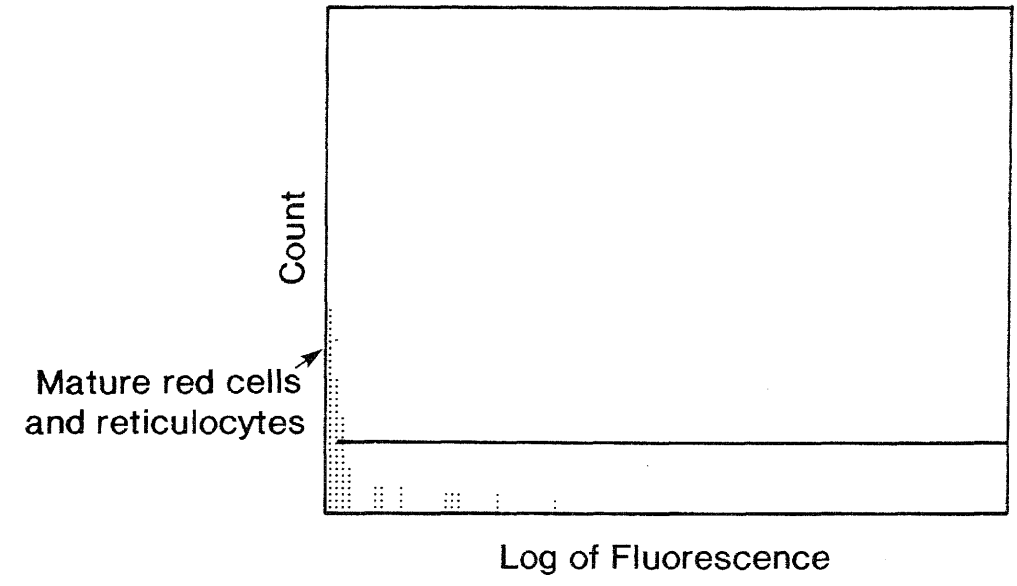


Fig. 8. Histograms representing count versus log of fluorescence - Control

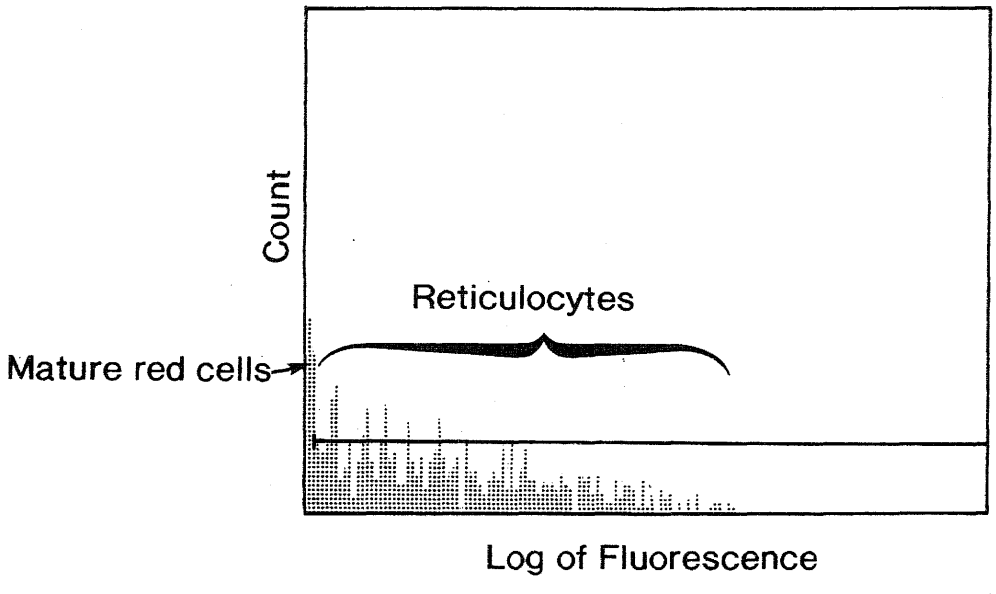


Fig. 9. Histograms representing count versus log of fluorescence - Test

5.4 Dedicated reticulocyte counter (RD)

Specimens were tested using the Sysmex R-3000 instrument (Sysmex-TOA Electronics company, Kobe, Japan), which uses a 10W argon laser light source which excites cells at 488 nm and emits light which is detected through a green filter. One hundred microliters of well mixed whole blood is sampled and stained with the fluorescent dye, auramine - O. Thirty thousand cells are counted per analysis.

Through computerization, gates are set for each sample to exclude white cells (nucleated cells) and platelets. The non-fluorescent mature red blood cells and fluorescent reticulocytes are then counted. The channels containing the fluorescent reticulocytes are divided into three to give the percentage of reticulocytes with low, moderate and high fluorescence. The red cell count is also measured and the absolute reticulocyte count calculated directly.

5.5 Study Design

5.5.1 Thiazole Orange Concentrations

Reticulocyte counts were tested on five specimens with thiazole orange concentrations of 1:10000, 1:5000 and 1:1000 in PBS by the RGP. These results were compared to those obtained by the RM and RD on the same samples, to determine which thiazole orange concentration correlated most closely.

5.5.2 **Incubation using RGP**

In order to determine the optimal time at which specimens should be analysed after incubation with thiazole orange, five samples were tested at various incubation times. The reticulocyte count as well as the mean channel number (MCN) (see chapter 3) value were assessed.

A Wilcoxon test was used to determine whether there was a statistically significant difference between the results obtained at different incubation times.

5.5.3 **Accuracy**

It is well known that the accuracy of reticulocyte counting is difficult to determine as there is no reference method for performing this test. In order to address this problem, a temporary reference material was made. A sample with a high (37,94%) reticulocyte count was counted by the RM. A paper diaphragm with a 4 X 4mm^2 square cut out of it was inserted into the microscope eyepiece to improve the accuracy of reticulocyte counting. Nine thousand and seventeen red cells were counted, which gives a 95% confidence interval for the percentage of reticulocytes of 36,94-38,94, based on the formula $Sp = \sqrt{\frac{p(1-p)}{m}}$ (see chapter 1).

This specimen was then diluted with a sample with a reticulocyte count of 0,51% (9044 red cell counted) which gives a 95% confidence interval of 0,36-0,66. These samples were diluted at different concentrations to obtain progressively lower reticulocyte counts (see Table 8). The reticulocyte counts of the reference preparation were then calculated, based on the volume of each sample used.

The precision of pipetting was also established to determine the reliability of the calculated reference values based on sample dilutions. Using distilled water, the volume of fluid dispensed 10 times by each pippette used was weighed and the coefficient of variation calculated, as a measure of precision. Also, the mean value of the amount of fluid dispensed by each pippette was used to calculate reticulocyte values based on sample dilutions.

The above preparations were then analysed by RM, RGP and RD to determine which method most closely approximated the reference reticulocyte counts. Different PMT's were used on the RGP as well to determine which gave the most accurate results. Statistically, comparisons of the different methods with the reference method were performed, using linear regression analysis.

5.5.4 **Precision**

Samples with a low, intermediate and elevated reticulocyte count were each tested 10 times by RGP and RD to determine the precision of reticulocyte counting by these methods. These same samples were also tested by RM by 4 different observers, each observer counting each of the 3 samples 10 times. The observers were blind as to the number of different specimens being counted. The coefficients of variation were calculated as a measure of the precision of the different methods.

5.5.5 **Correlation between RM, RGP and RD**

39 samples of whole blood were tested by RM, RGP and RD, to establish the correlation between the different methods. Linear regression analyses were used to compare RM with RGP, RM with RD, and RGP with RD. Different PMT's were also used on the RGP to determine which level correlated most closely with the RM and RD.

5.5.6 **Comparison of MCN and HFR**

The MCN value on the general purpose flow cytometer and the HFR value on the Sysmex R-3000 instrument were compared on the same 65 specimens using linear regression analysis to see if there is a good agreement between these measurements.

5.5.7 **Interesting cases**

Examples of cases which demonstrated certain interesting points were analysed.

5.5.7.1. A sample with a malaria parasitaemia of 20% was tested by RM, RGP and RD.

5.5.7.2 Blood from a patient with Chronic Lymphocytic Leukaemia(CLL) was analysed by the 3 methods.

5.5.7.3 Reticulocyte counts were performed by the 3 methods on a patient with sickle cell disease in crisis.

5.5.7.4 Reticulocyte counts were assessed by the 3 methods on a blood sample containing Pappenheimer bodies.

5.5.7.5 Blood samples from a patient with Chronic Myeloid Leukaemia (CML) were analysed by the three methods.

5.5.7.6 Serial reticulocyte counts and MCN (RMI) estimations were used to determine the response of a patient with a severe megaloblastic anaemia to haematinic therapy, using an Epics Profile I (Coulter Electronics, Hialeah, FL) general purpose flow cytometer.

5.5.7.7 A patient with a haemolytic anaemia was also assessed using the reticulocyte count and MCN estimations on an Epics Profile I flow cytometer.

CHAPTER 6

RESULTS

Results

NOTE: The derived data are represented in this section, while the raw data on which these results are based are located in the appendix.

6.1 Thiazole orange concentrations

Reticulocyte counts were performed using a general purpose flow cytometer (RGP) with different thiazole orange concentrations. The samples were incubated for one hour prior to analysis. These results were compared with those obtained on the same samples using the manual microscopic method (RM) and a dedicated flow cytometric reticulocyte counter (RD). A thiazole orange concentration of 1:10 000 gave reticulocyte results which correlated most closely with RM and RD, especially at low reticulocyte counts. Therefore this concentration was used for all further studies.

6.2 Incubation times using RGP

Blood samples were incubated for different time periods in thiazole orange. The Wilcoxon test was used to establish whether the reticulocyte results at different incubation times were significantly different from one another or not. There was no significant difference between results obtained at incubation times of between 30 mins and 6 hours ($P > 0,05$). However, results obtained at 15 mins and 24 hours were significantly different from those obtained at incubation times of between 30 mins and 6 hours. ($P < 0,05$) (Fig. 10)

The effect of incubation time on the mean channel number (MCN) value was assessed as well. No significant difference was found between results at incubation times of between 1 and 6 hours ($P > 0,05$). There was a significant difference between the values at time of less than 1 hour, when compared with those at 1 hour or more ($P < 0,05$). Also, at 24 hours there was a significant difference from the values obtained after 6 hours of incubation (Fig. 11).

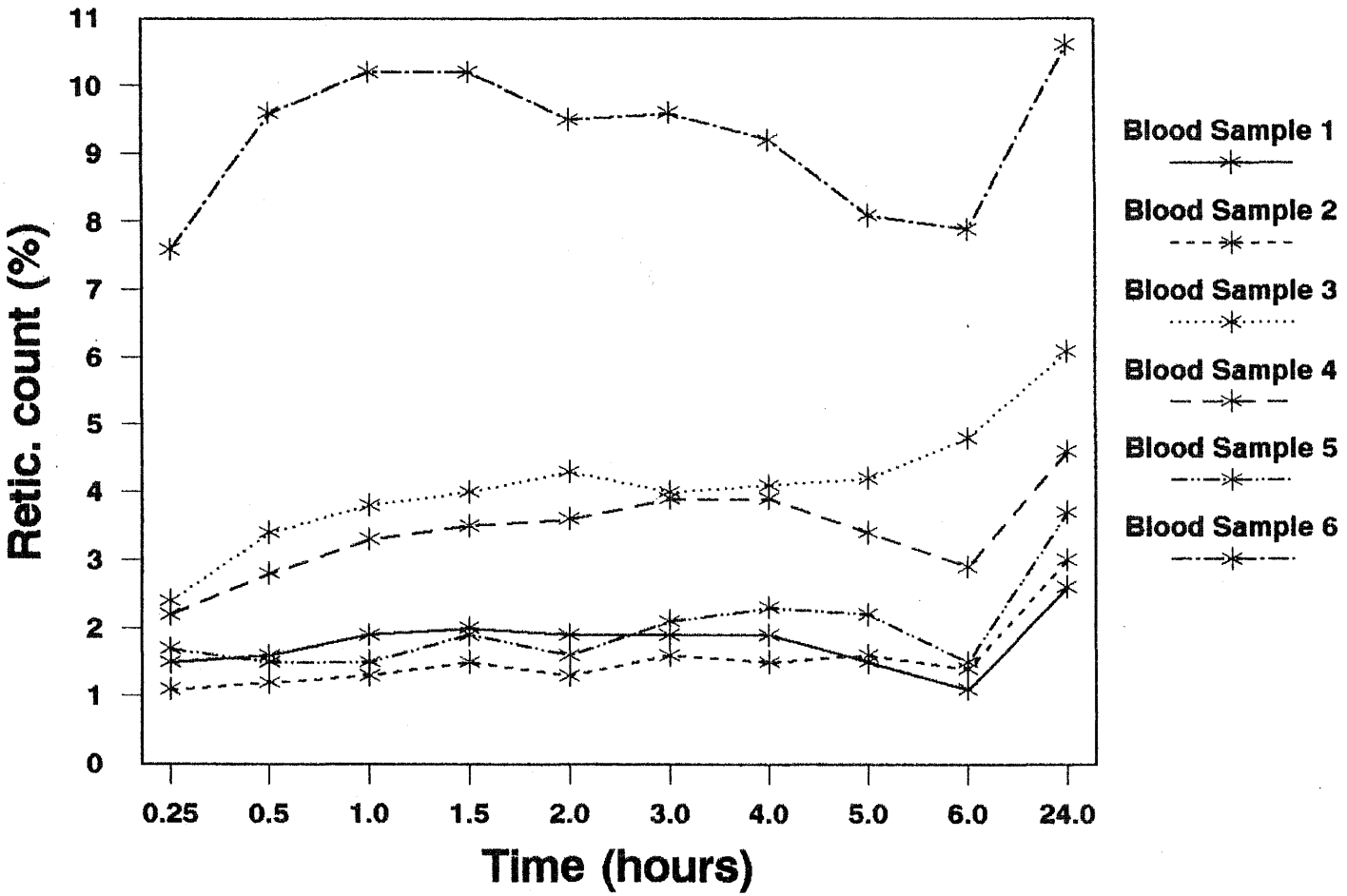


Fig.10. Effect of different incubation times on reticulocyte results

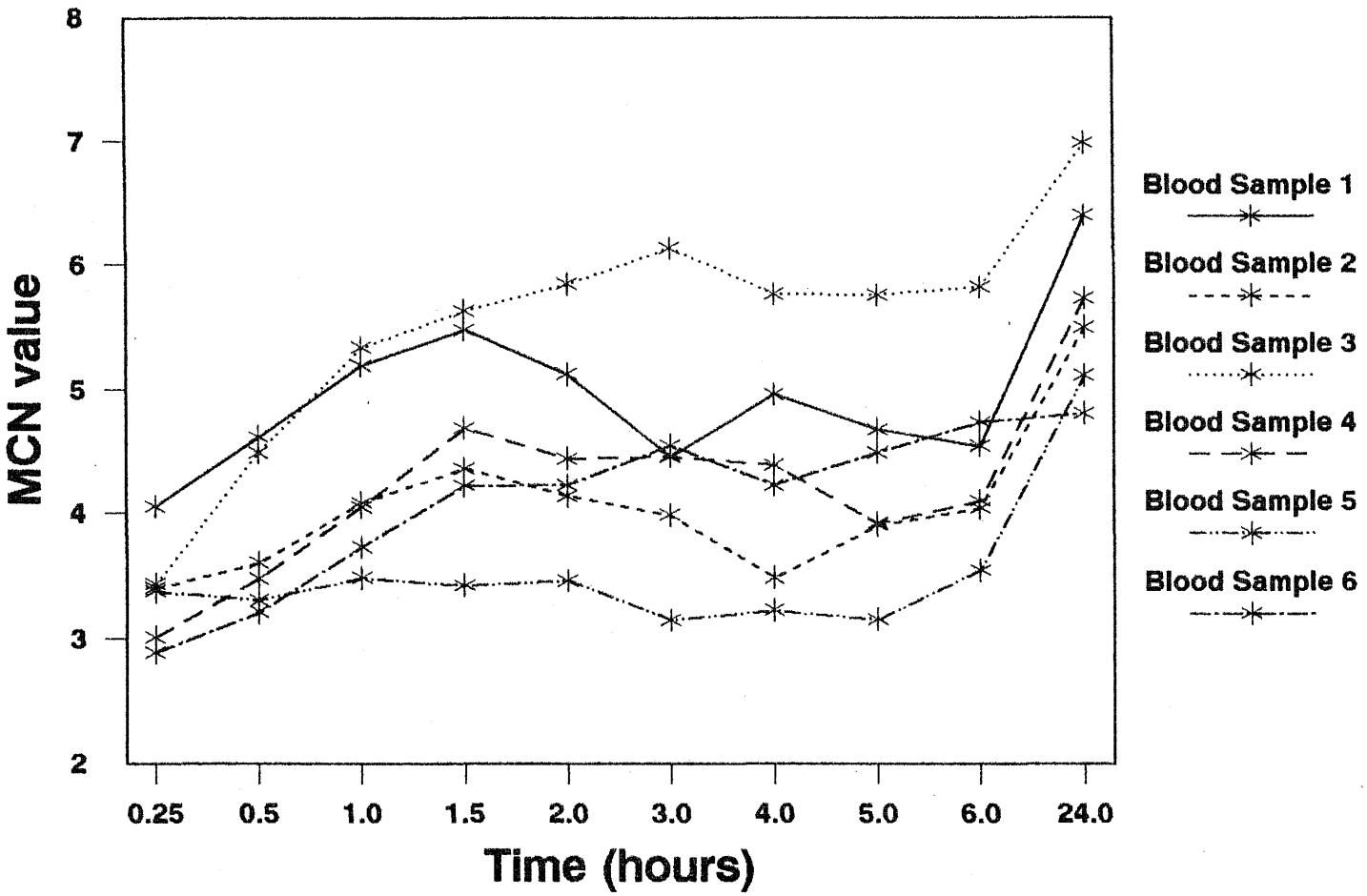


Fig.11. Effect of different incubation times on MCN values.

6.3 Accuracy

A blood sample with a high reticulocyte count and a sample with a low reticulocyte count were diluted at different concentrations, using a pippette, to obtain a reference preparation. The precision of pipetting was good, with CV's all $\leq$ 6,7% . Therefore the calculated reticulocyte counts based on sample dilutions were considered to be reliable. The range of dilutions covered reticulocyte counts from 37,94% to 0,51%.

Reticulocyte counts were performed using RM, RGP and RD, and compared with the reference values. Linear regression was used to compare the different methods of reticulocyte analysis with the reference method.

The RGP method correlated very well with the reference method, with very high R-square values at all PMT's (The PMT values represent different sensitivities of the instrument for detecting fluorescence). At a PMT setting of 975, the results correlated exceptionally well with the reference values, with only a very slight overestimation of reticulocyte counts. A PMT of 1050 also gave very good results, with a small overestimation of counts. (Table 3 and Figs. 12-15). Thus the instrument appears to be very accurate at both PMT values. A PMT setting of 975 was chosen for all further studies.

The Sysmex R-3000 instrument results did not correlate well with the reference method, with an R-square value of only 0,881 (Table 3 and Fig. 16). Also, the instrument was overestimating counts at one reticulocyte level and underestimating at another level, which cannot be corrected for.

The standard manual method had a very good correlation with the reference method, although not as good as the general purpose flow cytometer, based on

the slope value which is closer to unity than that of the general purpose flow cytometer. (Table 3 and Fig. 17). It is noteworthy that the manual reticulocyte analyses were performed by a very experienced technologist. This factor must be taken into account, as the results may not always be as good, especially if the analyses were performed by an inexperienced person.

Table 3. Results of Linear Regression analyses comparing reticulocyte counts by flow cytometric and manual methods with reference values.

Method	Slope	Intercept	R-square
RGP 900	0,81	1,27	0,996
RGP 950	0,92	1,57	0,998
RGP 975	1,03	1,11	0,999
RGP 1050	1,00	1,44	0,997
RM	1,09	0,21	0,995
RD	0,80	2,99	0,881

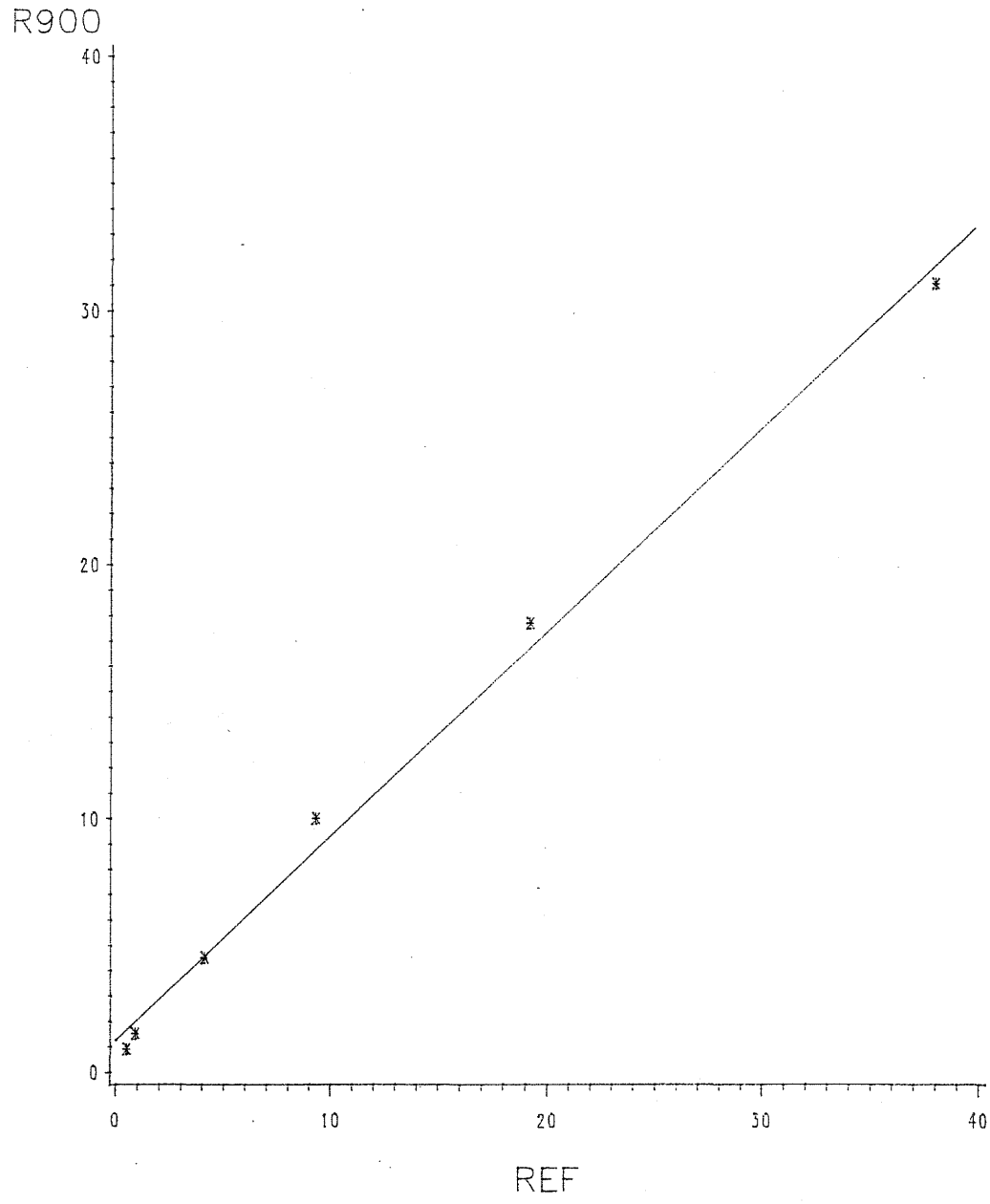


FIG.12 LINEAR REGRESSION ANALYSIS OF RGP (PMT 900) VERSUS THE REFERENCE VALUES

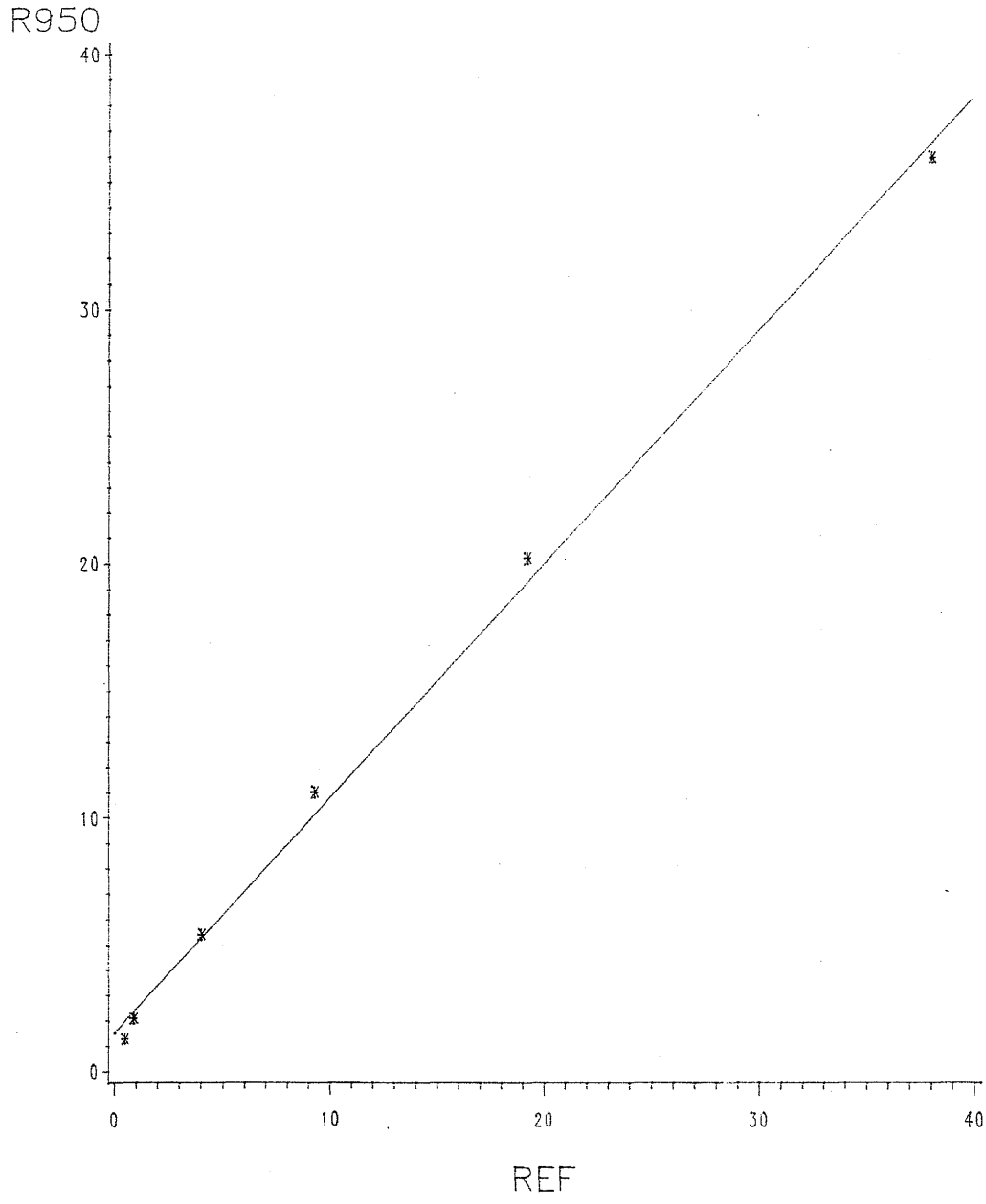


FIG.13 LINEAR REGRESSION ANALYSIS OF RGP (PMT 950) VERSUS THE REFERENCE VALUES

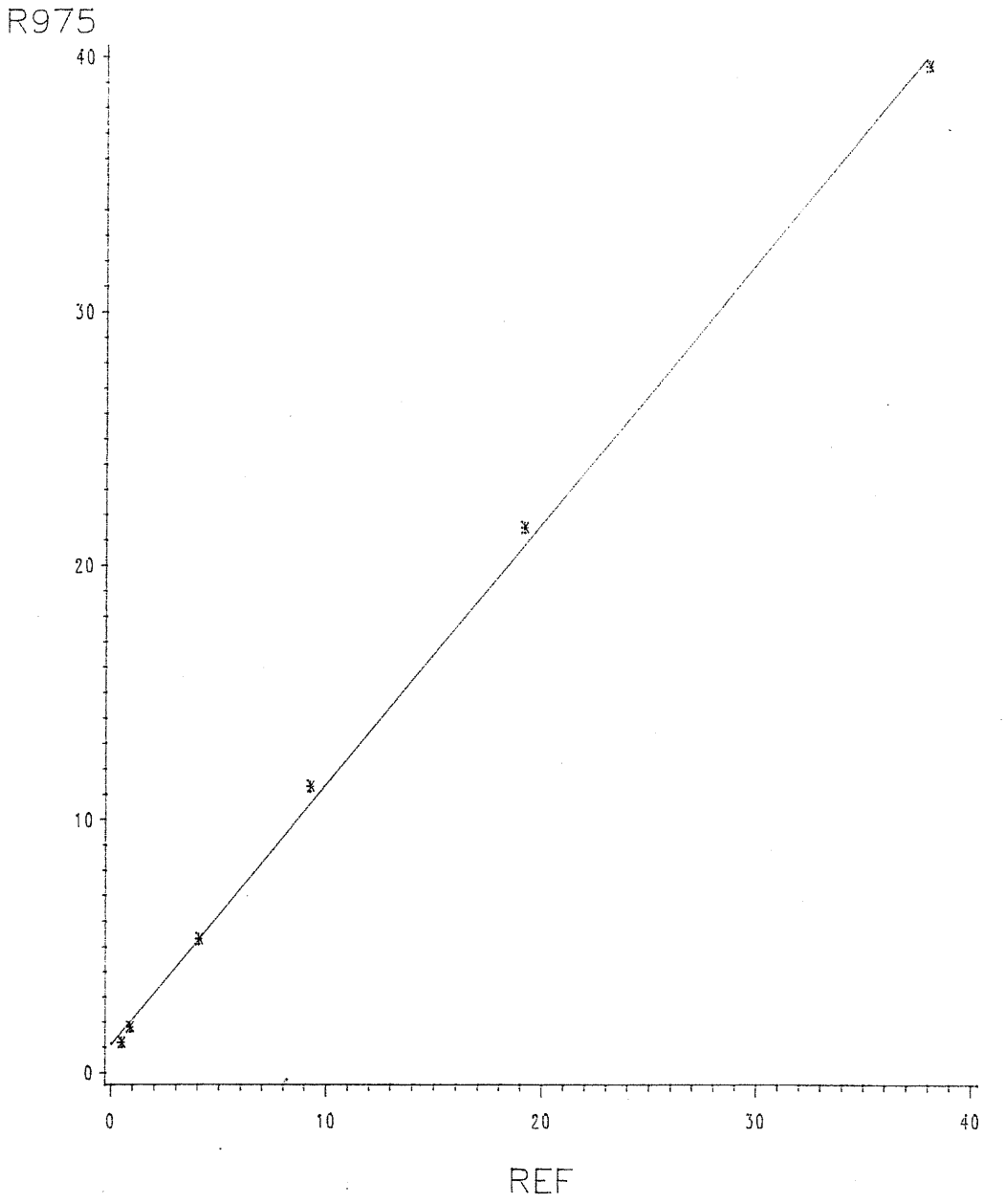


FIG.14 LINEAR REGRESSION ANALYSIS OF RGP (PMT 975) VERSUS THE REFERENCE VALUES

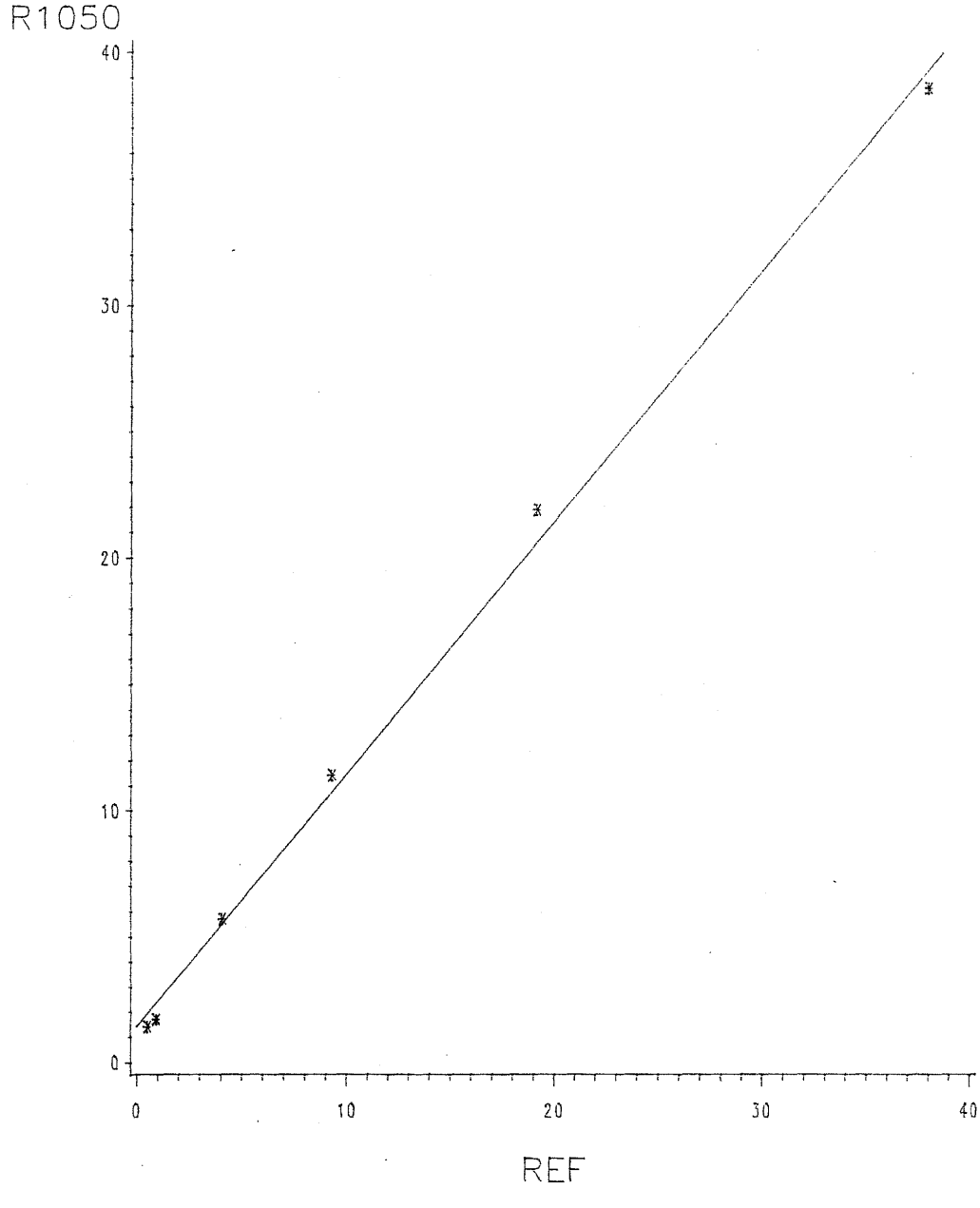


FIG.15 LINEAR REGRESSION ANALYSIS OF RGP (PMT 1050) VERSUS THE REFERENCE VALUES

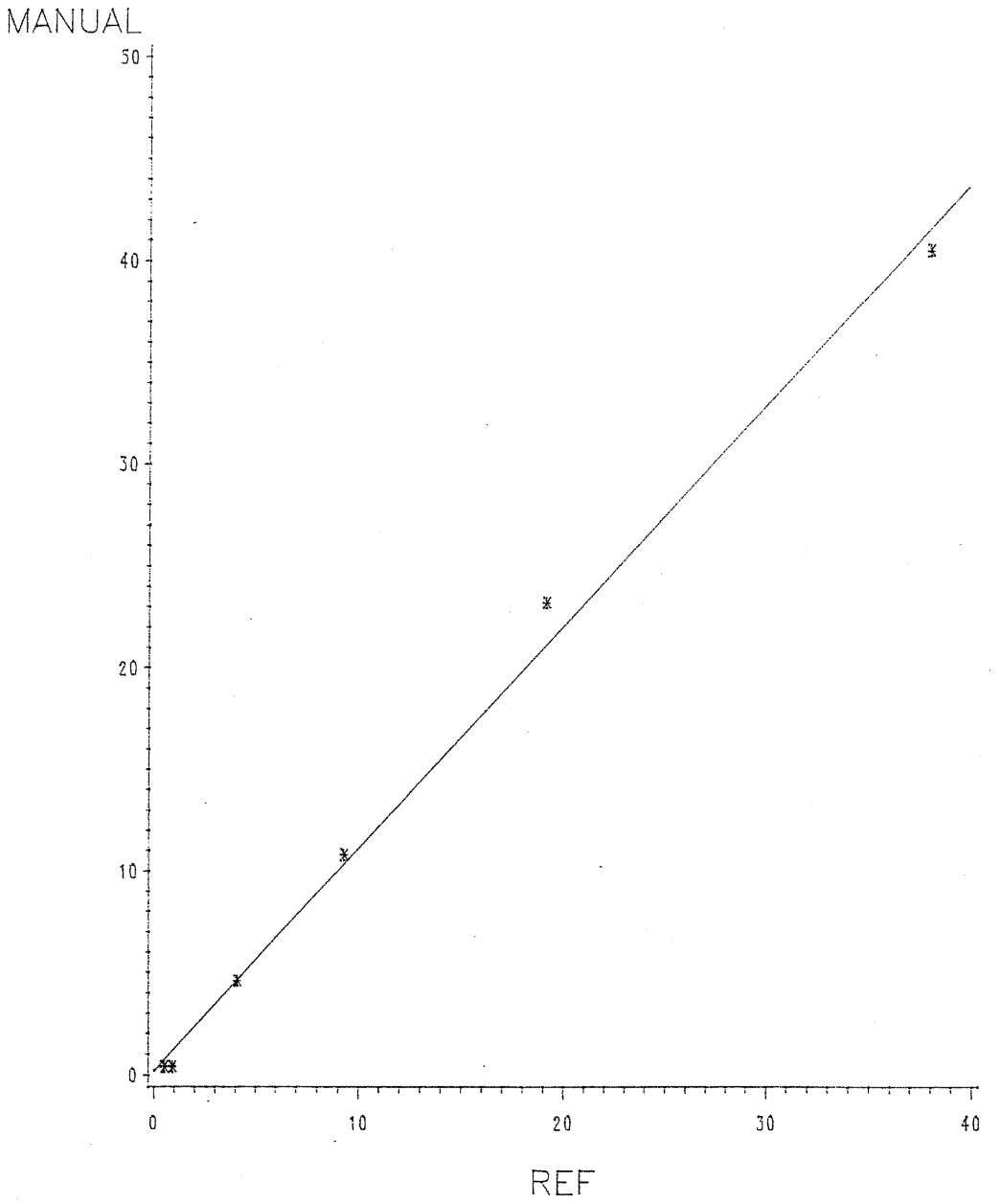


FIG. 16 LINEAR REGRESSION ANALYSIS OF RM VERSUS THE REFERENCE VALUES

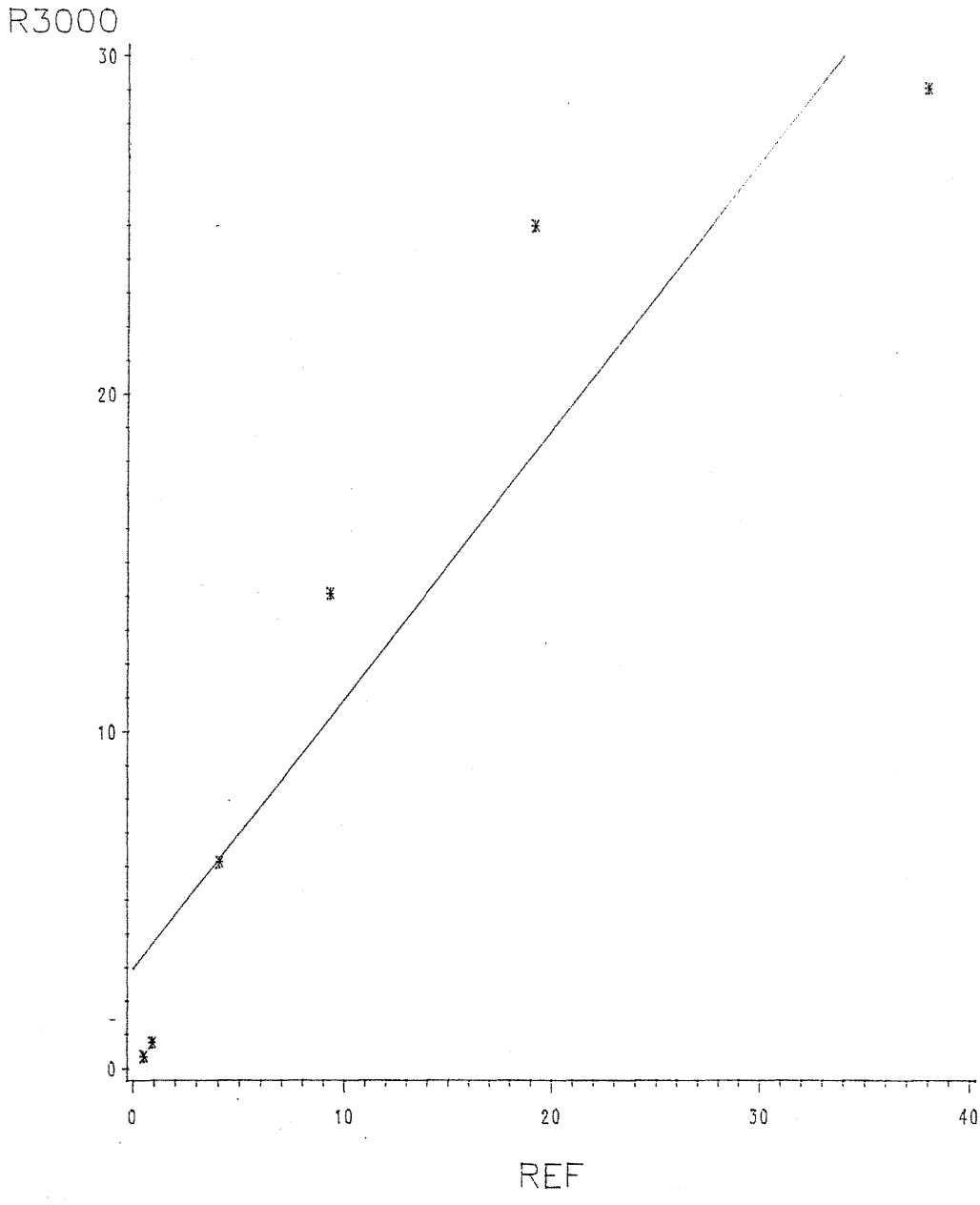


FIG.17 LINEAR REGRESSION ANALYSIS OF RD VERSUS THE REFERENCE VALUES

6.4 Precision of reticulocyte counting

The precision with both the RGP and RD was better than the intraobserver variation with the RM at very low, intermediate and elevated reticulocyte counts. At very low reticulocyte counts, the precision of the Sysmex R-3000 instrument was very good. However, with the Epics profile II, precision was not very good at low counts (although still better than the manual method). This is most likely due to problems in setting the negative control, based on an unstained sample. At higher reticulocyte counts however, the precision of the Epics profile II was better than that of the R-3000 instrument. (Fig. 18).

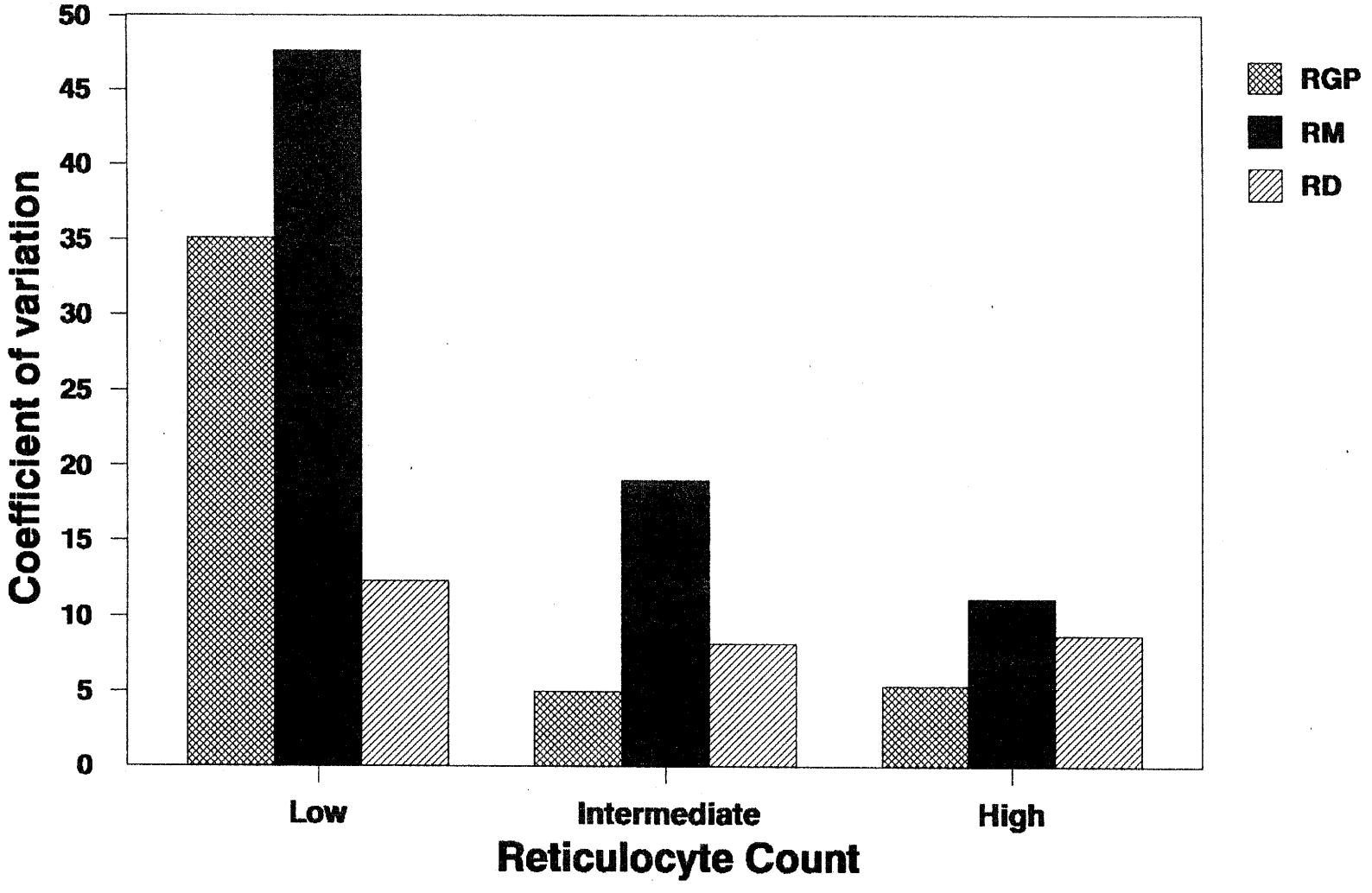


Fig.18. Precision of Reticulocyte Counting

6.5 Correlation between RM, RGP and RD

Reticulocyte analyses on 39 blood samples were performed by the three different methods. Reticulocyte counts ranged from 0 to $\pm$ 25%.

The RM, RD and RGP correlated well with one another, with R-square values all above 0,9. (Table 4 and Figs. 19-25).

Table 4. Results of linear regression analyses

Methods	Slope	Intercept	R-square
RGP 950 vs RD	0,90	0,31	0,925
RGP 975 vs RD	0,94	0,54	0,925
RGP 1000 vs RD	1,02	0,62	0,926
RM vs RD	1,07	0,81	0,901
RGP 950 vs RM	0,79	-0,12	0,918
RGP 975 vs RM	0,83	0,09	0,921
RGP 1000 vs RM	0,89	0,16	0,909

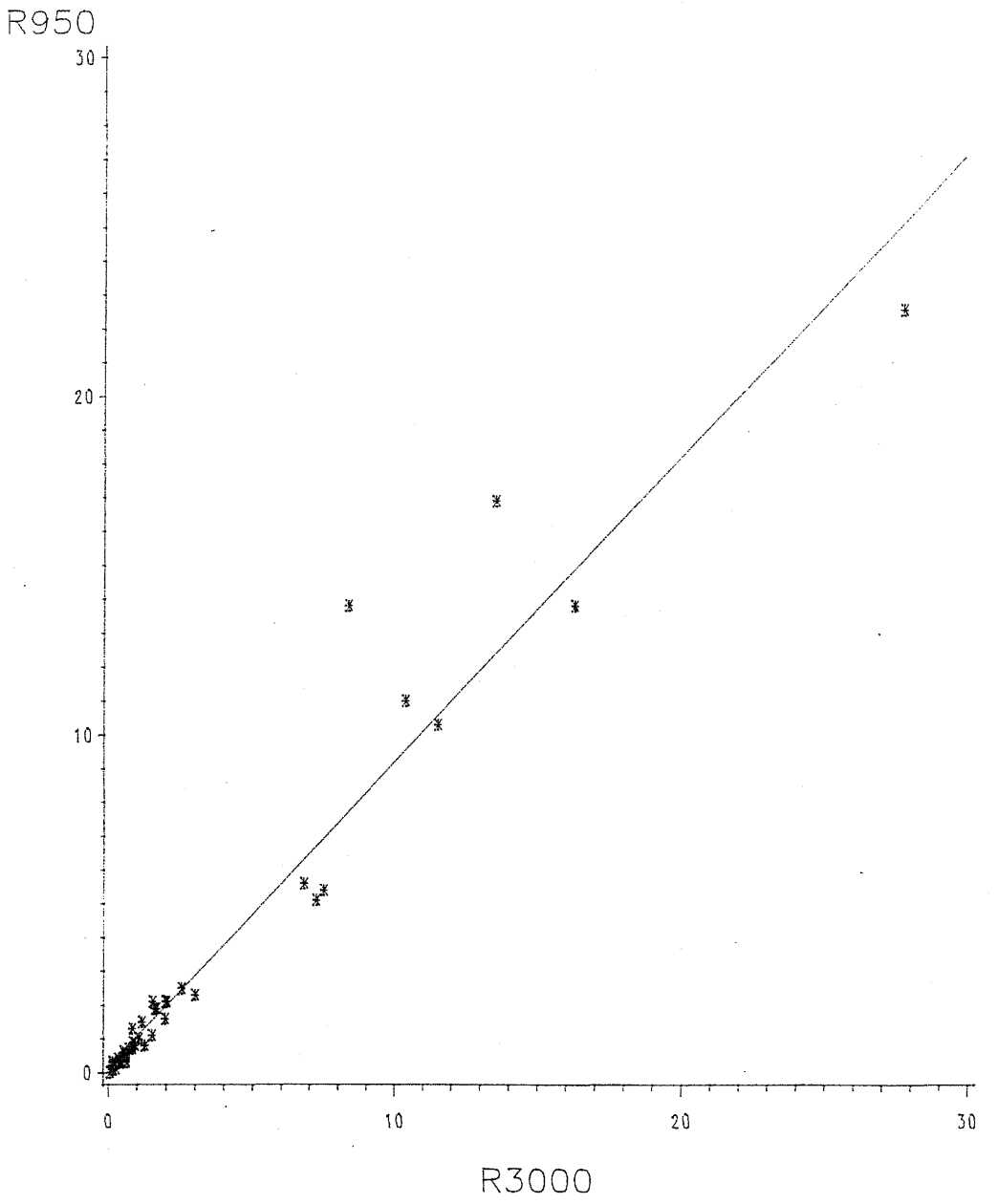


FIG.19 LINEAR REGRESSION ANALYSIS OF RGP (PMT 950) VERSUS RD

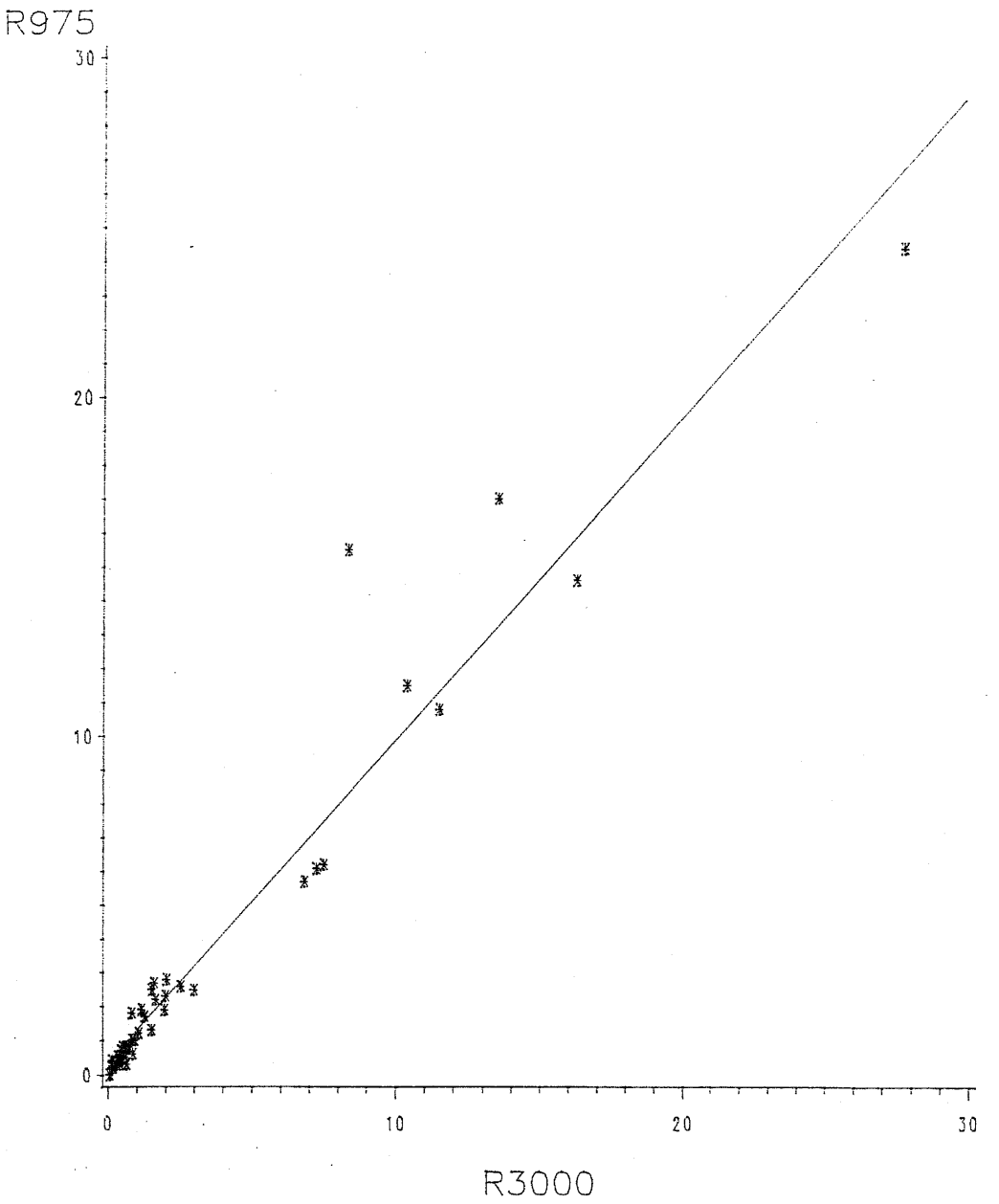


FIG. 20 LINEAR REGRESSION ANALYSIS OF RGP (PMT 975) VERSUS RD

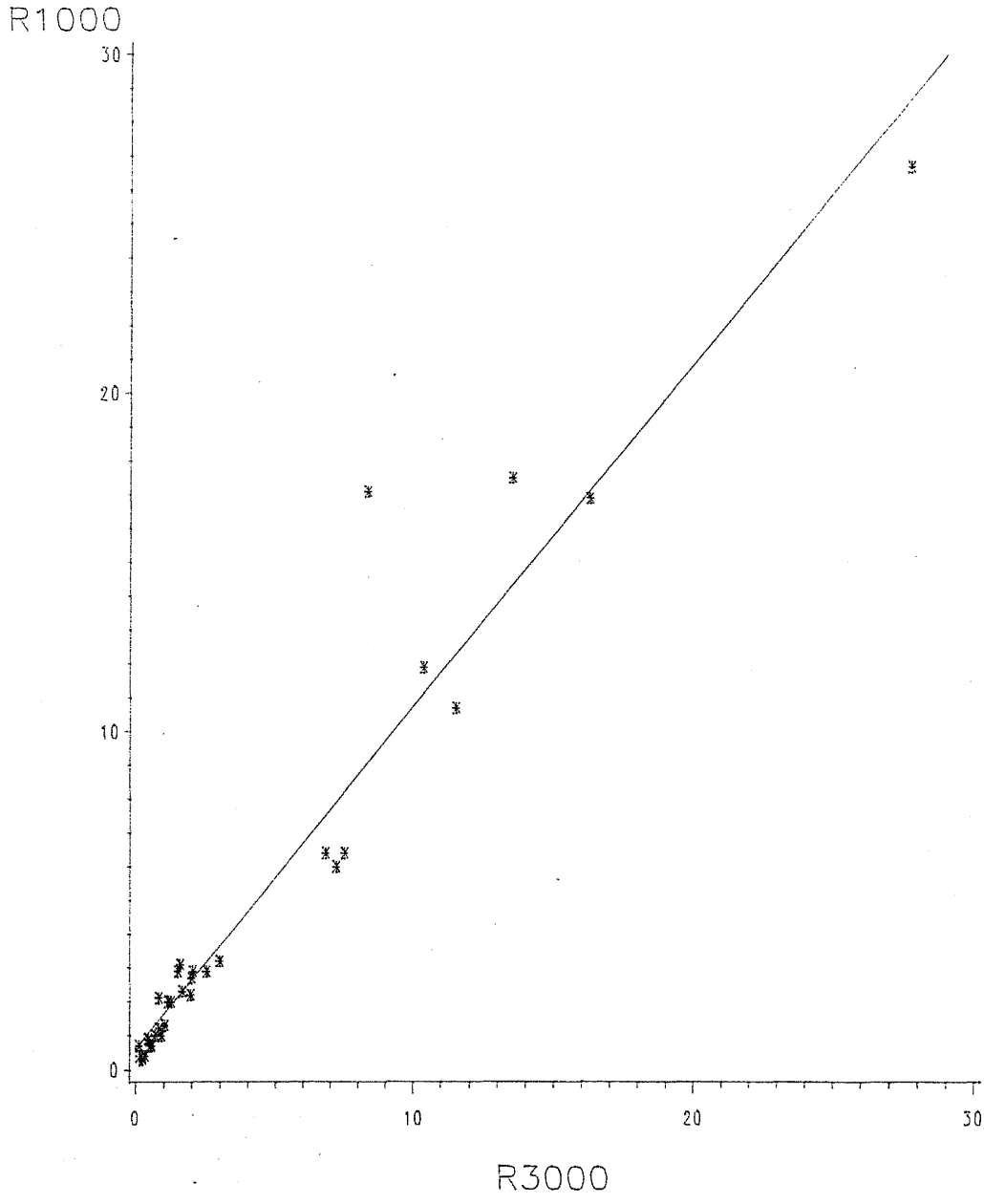


FIG.21 LINEAR REGRESSION ANALYSIS OF RGP (PMT 1000) VERSUS RD

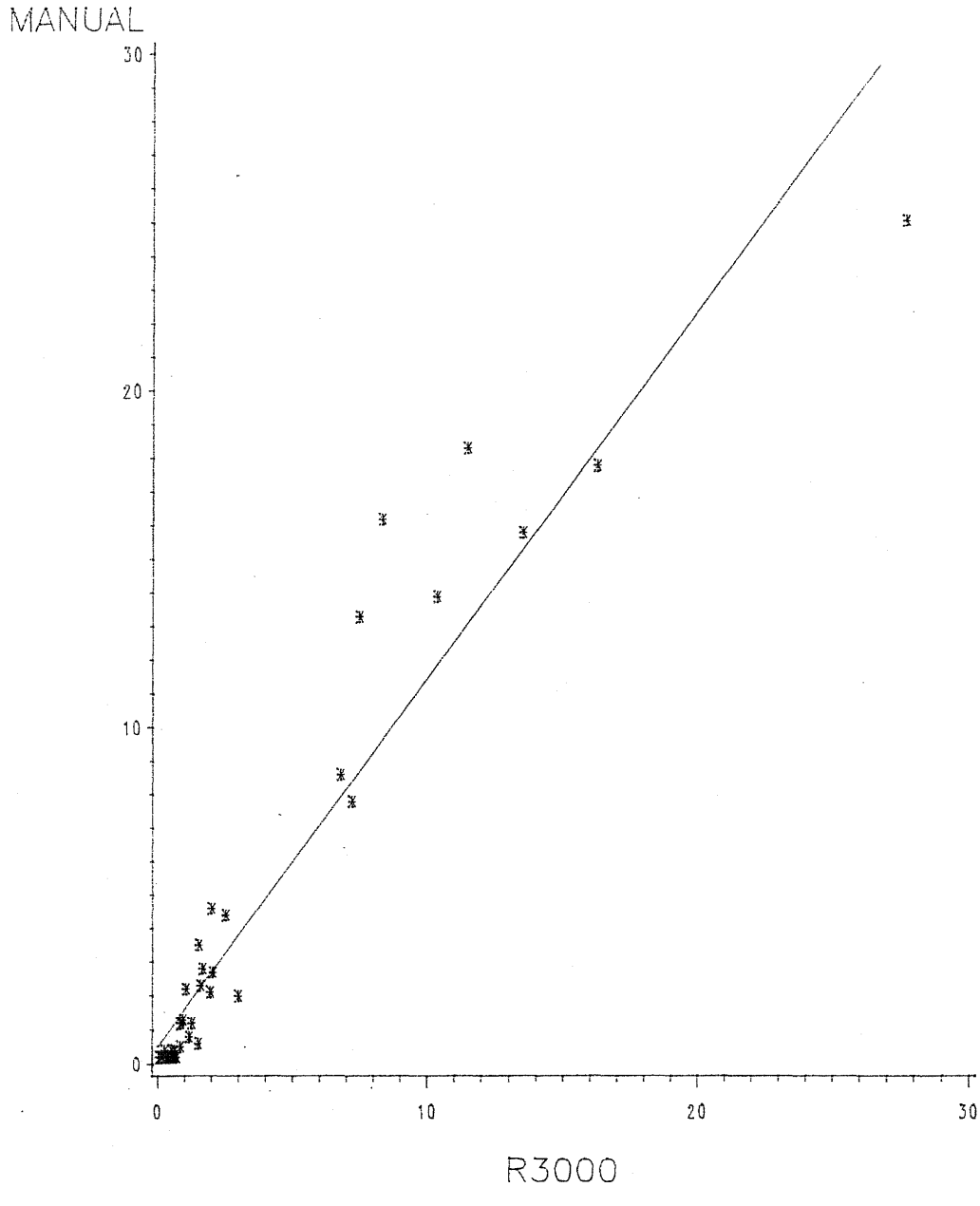


FIG. 22 LINEAR REGRESSION ANALYSIS OF RM VERSUS RD

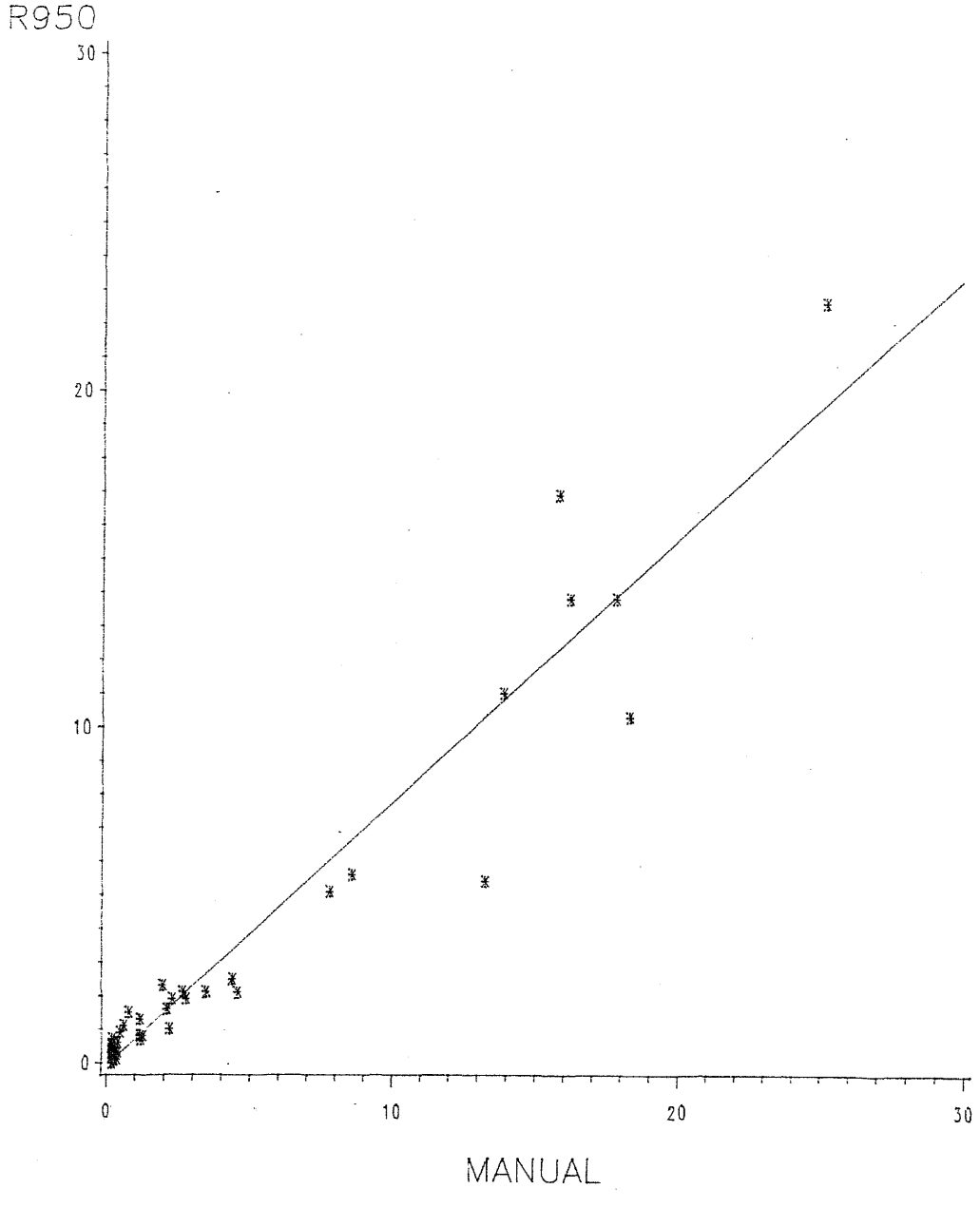


FIG.23 LINEAR REGRESSION ANALYSIS OF RGP (PMT 950) VERSUS RM

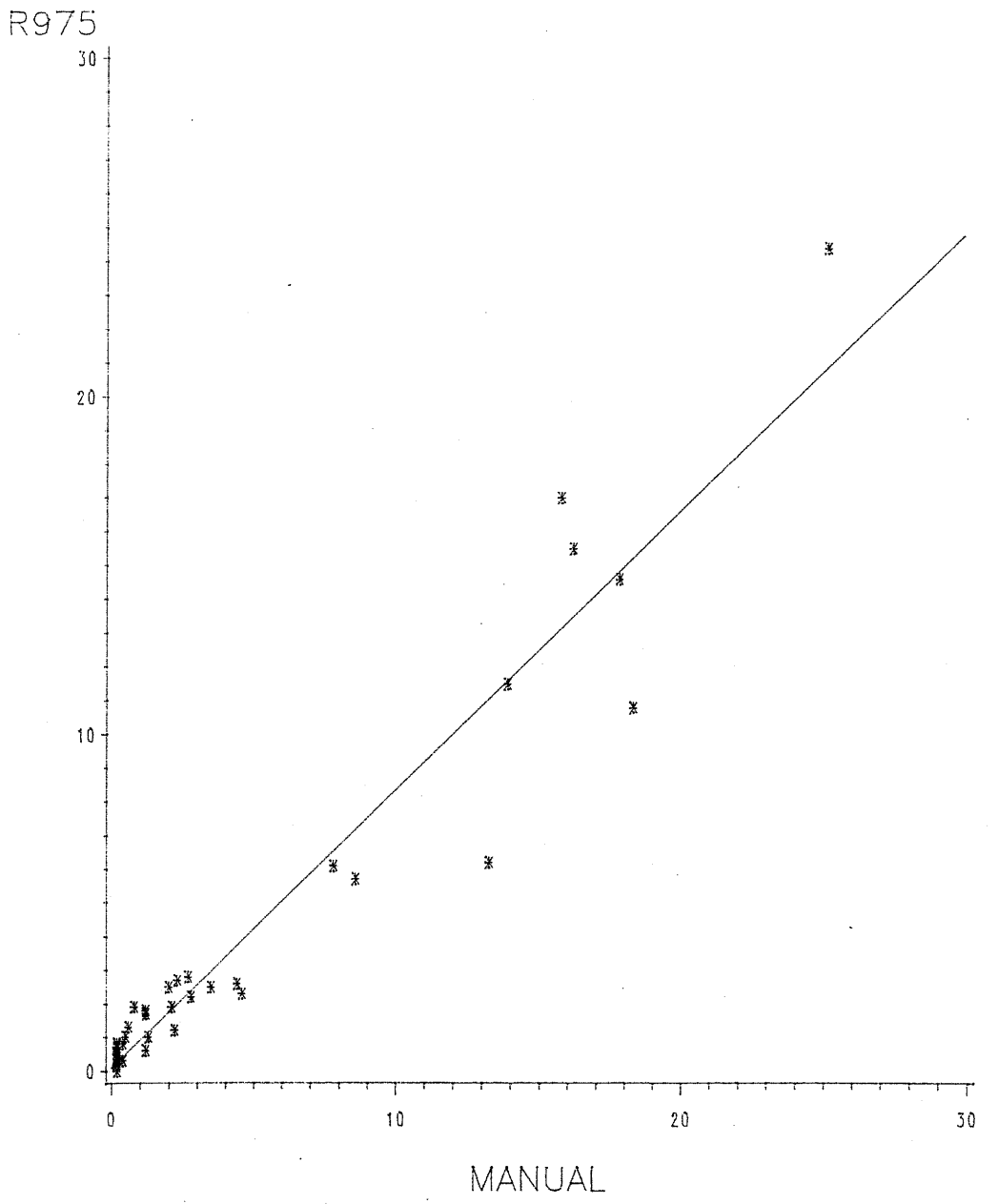


FIG.24 LINEAR REGRESSION ANALYSIS OF RGP (PMT 975) VERSUS RM

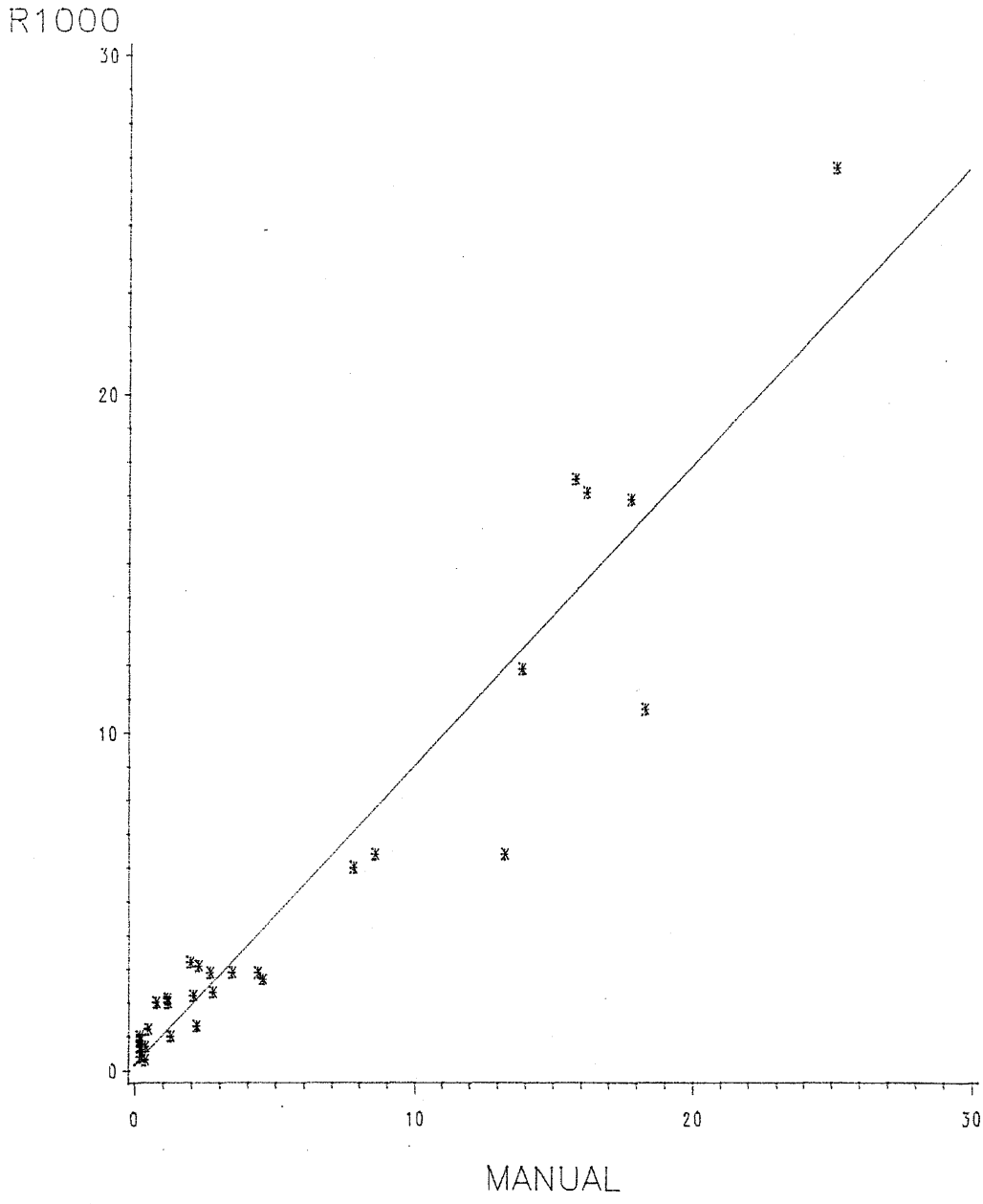


FIG.25 LINEAR REGRESSION ANALYSIS OF RGP (PMT 1000) VERSUS RM

6.6 Comparison of MCN and HFR

These two measurements correlated very poorly, with an R- square value of 13,58 (Fig. 26). This is to be expected, as they are measuring different variables.

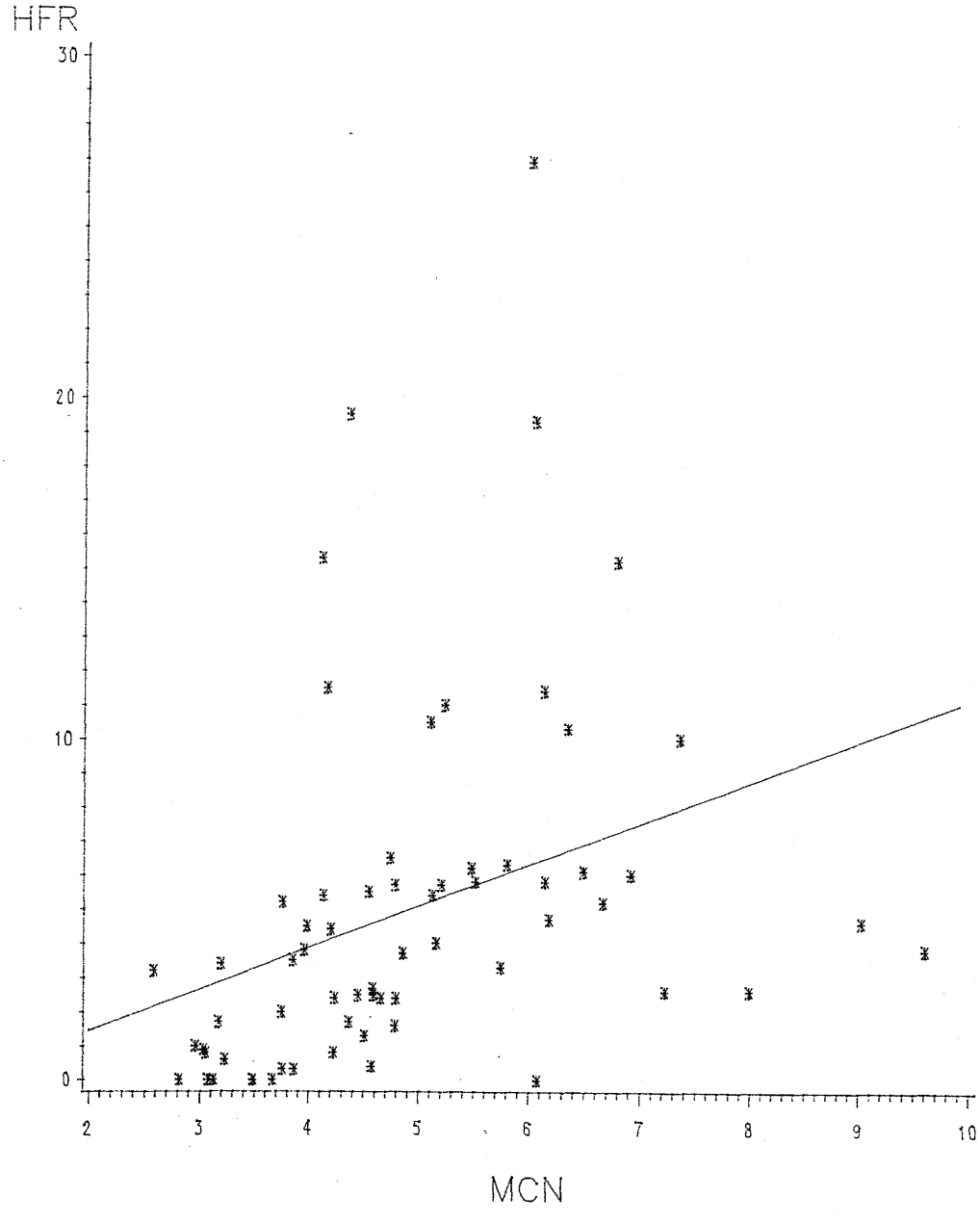


FIG.26 LINEAR REGRESSION ANALYSIS OF MCN VERSUS HFR VALUES

6.7 Interesting cases

6.7.1 *Malaria*

The manual reticulocyte count on the blood specimen with a 20% malaria parasitaemia was 0,4% (Fig. 27). With the RGP, it was 17,9% (control 0,1) (Fig. 28) and with the RD, 11,48%. Both flow cytometric instruments were thus not able to discriminate between reticulocytes and parasitized red blood cells. The parasitized red cells are represented in the lower fluorescence channels on both instruments. This could constitute an advantage, as it is likely that such cases would be flagged for morphological assessment.

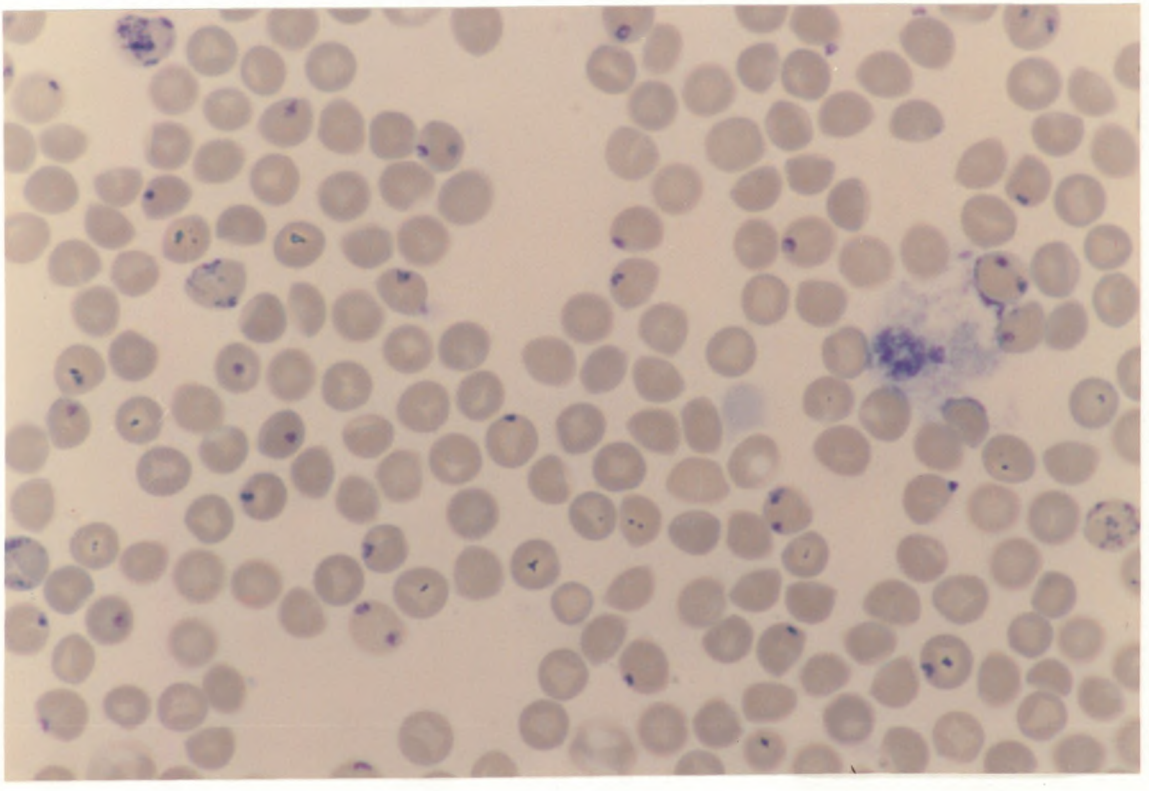
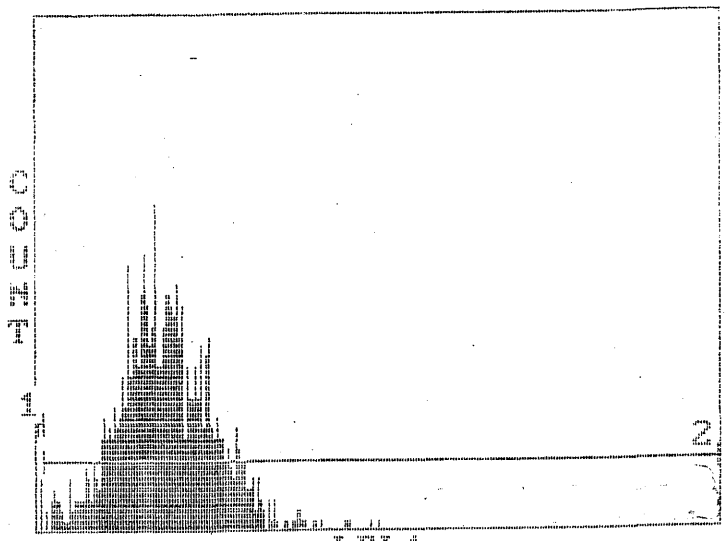


Fig 27. New methylene blue reticulocyte stain on blood from patient with malaria.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.095	16394	82.0	1.038	1.028	30.1
2	1.095	1023.	3606	18.0	3.695	1.697	2.17
3	65.31	1023.	0	0.0			

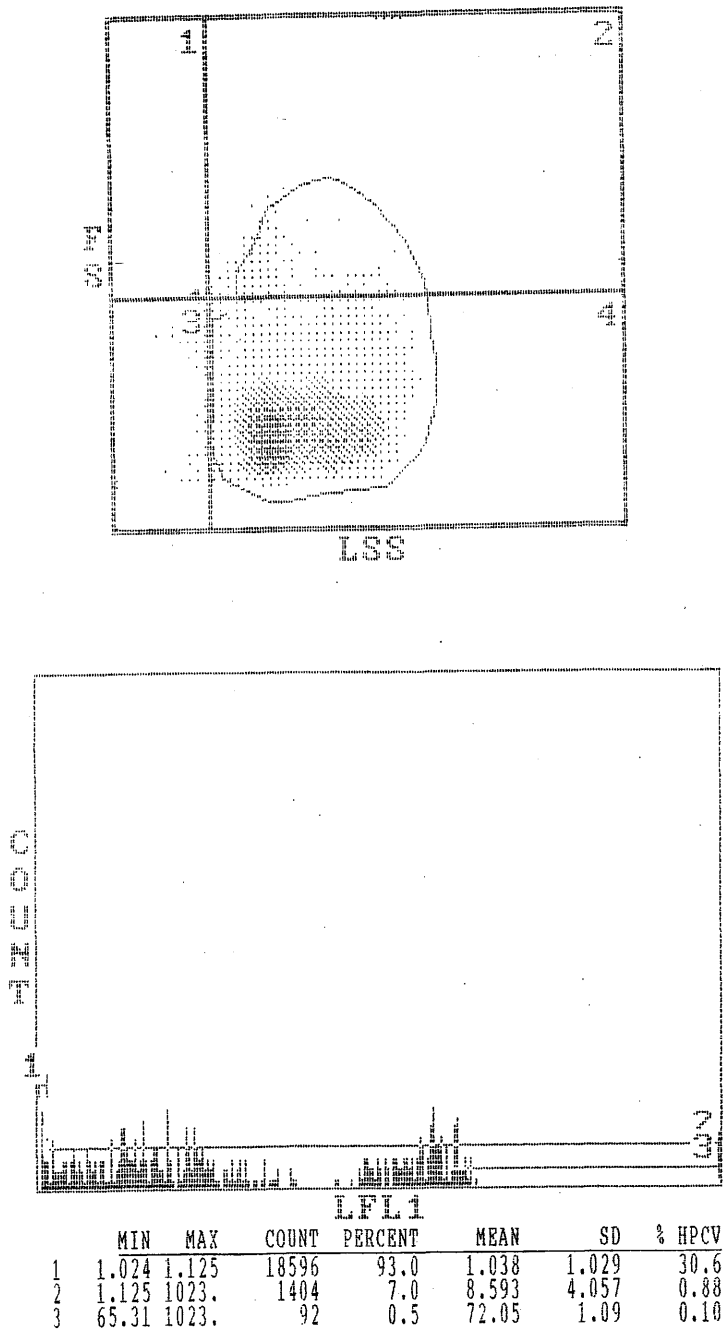
Fig. 28. Histogram showing Malaria Parasitaemia

Gated area number 1 represents mature red blood cells which are non-fluorescent and area number 2 includes reticulocytes and all cells which take up the fluorescent dye (in this case, parasitized red blood cells).

LFL1 = Log of fluorescence

6.7.2 *Chronic lymphocytic leukaemia (CLL)*

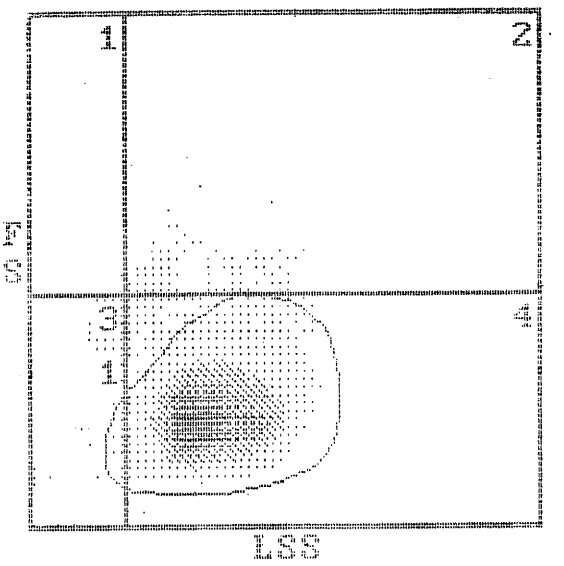
The manual reticulocyte count of the patient with CLL was 6,6%. Using the RGP, a reticulocyte count of 7,0% (control 0) was obtained (Fig. 29A). The bitmap on the first histogram was altered to exclude the lymphocytes (Fig. 29B) which changed the reticulocyte count to 5,3%, which is closer to the reticulocyte count obtained using the Sysmex R-3000 instrument, which was 5,62%. From Fig. 29, one can also see that the most highly fluorescent cells are excluded when the bitmap is changed to exclude lymphocytes. These highly fluorescent cells would represent the lymphocytes, which are nucleated and thus have a high DNA content.

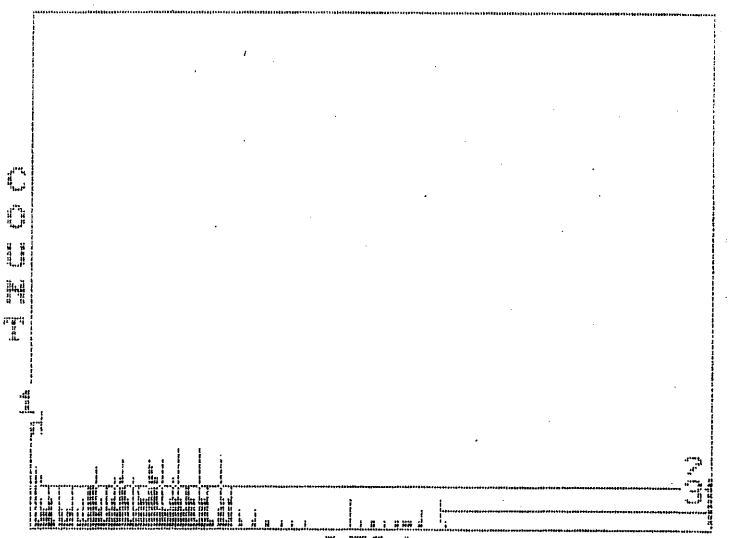


	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.125	18596	93.0	1.038	1.029	30.6
2	1.125	1023.	1404	7.0	8.593	4.057	0.88
3	65.31	1023.	92	0.5	72.05	1.09	0.10

Fig. 29A. Histograms from patient with CLL.

Red blood cells are bitmapped on the histogram representing forward scatter (FS) versus log of side scatter (LSS). Lymphocytes are situated very close to the red blood cells on this histogram, and therefore many were included in the bitmap. The cells included in the bitmapped area on the first histogram are shown on the second histogram, representing count versus log of fluorescence (LFL1). The fluorescent cells (reticulocytes and white blood cells) are included in the gated area number 2.





	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.125	18949	94.7	1.038	1.028	30.3
2	1.125	1023.	1051	5.3	3.523	2.346	1.21
3	65.31	1023.	7	0.0	71.50	1.08	0.21

Fig. 29B. Histograms from patient with CLL.

The bitmap on the first histogram is made smaller, to exclude most of the lymphocytes. On the second histogram, representing count versus log of fluorescence (LFL1), the peak of fluorescent white blood cells in the area gated number 2 has almost disappeared.

6.7.3 *Sickle Cell Anaemia*

The reticulocyte count using the RGP was 22,5%, (control 0,3) which is very similar to the counts obtained by RM and RD which were 25,1% and 27,69% respectively. (Fig. 30 and 31). There was therefore no exclusion of the sickled red blood cells from analysis by either flow cytometric instrument.

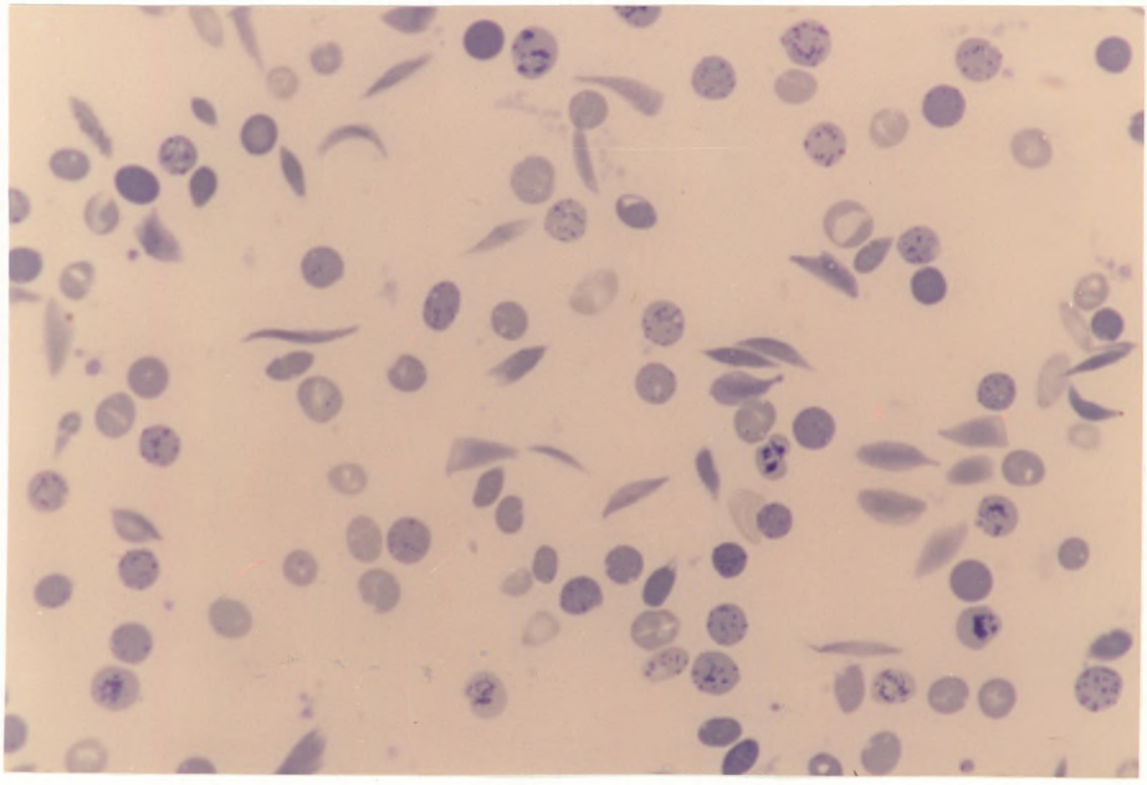
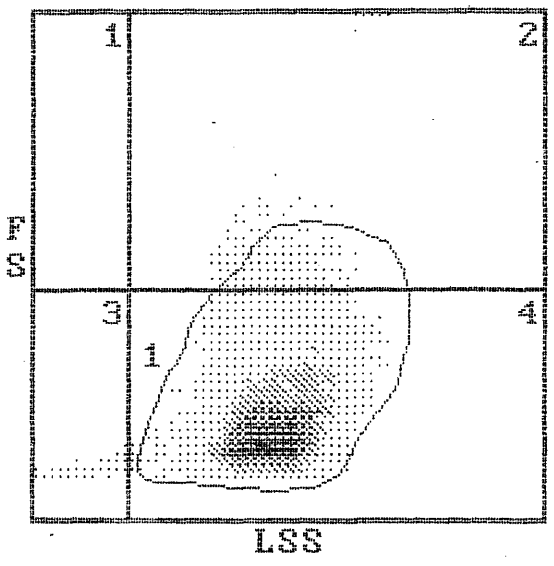
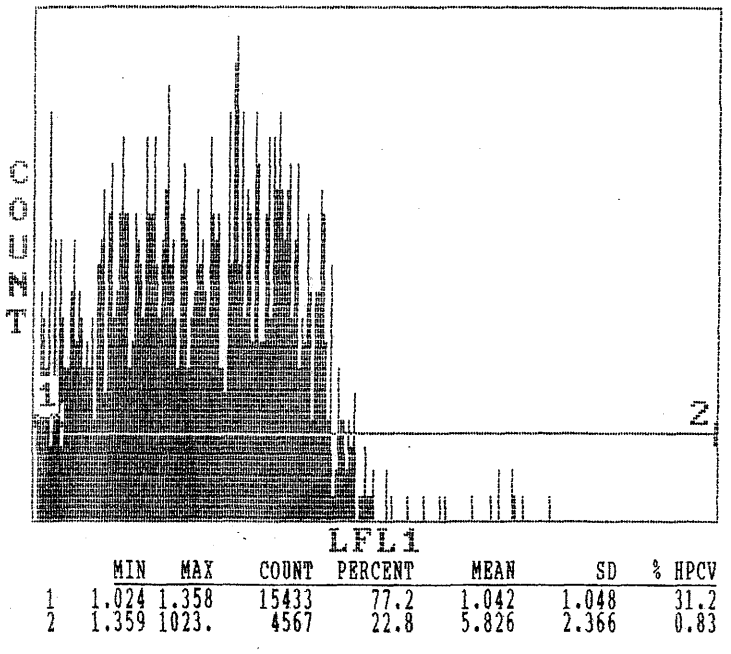


Fig. 30. New methylene blue reticulocyte stain of blood from a patient with Sickle Cell Anaemia.





	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.358	15433	77.2	1.042	1.048	31.2
2	1.359	1023.	4567	22.8	5.826	2.366	0.83

Fig. 31. Histograms showing reticulocyte analysis in patient with Sickle Cell Anaemia.

Red blood cells are bitmapped on the first histogram, representing forward scatter (FS) versus log of side scatter (LSS). These cells are displayed on the second histogram, representing count versus log of fluorescence (LFL1). The mature red blood cells are included in the area gated number 1 and reticulocytes are shown in the area gated number 2.

6.7.4 *Pappenheimer bodies*

The blood sample with Pappenheimer bodies had a manual reticulocyte count of 0,2%. (Fig 32). The inclusions were confirmed to be iron granules by staining the slides with Prussian blue (Fig. 33). The RGP level was 3,3% (control 0,2) (Fig. 34) whereas the RD count was 0,26%. So the RGP method cannot distinguish between red cells containing Pappenheimer bodies and reticulocytes, whereas this problem did not occur with the RD.

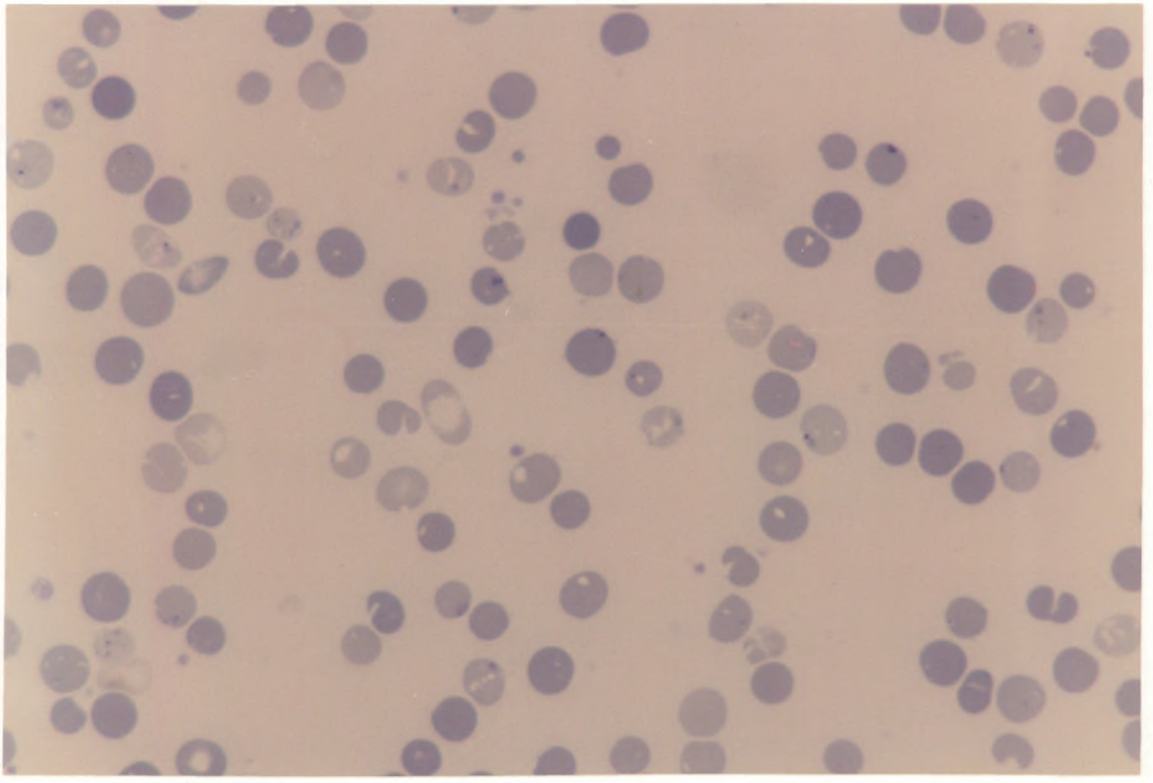


Fig. 32. New methylene blue stain of blood containing Pappenheimer bodies.

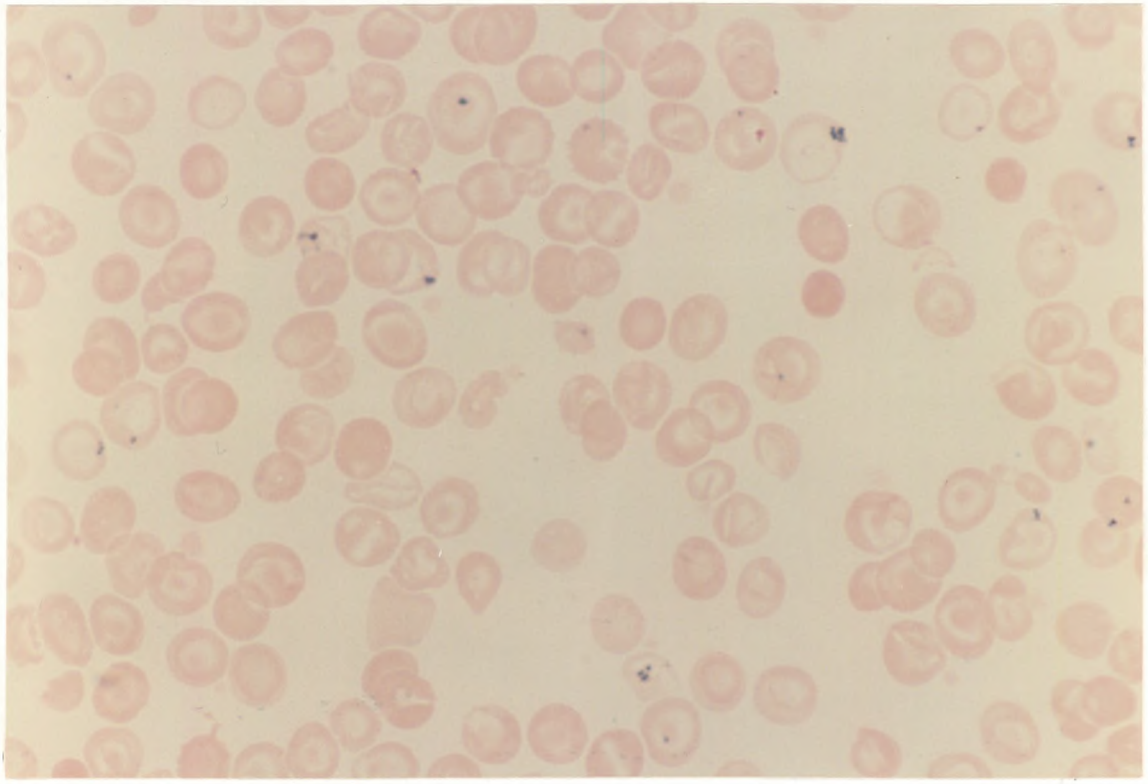
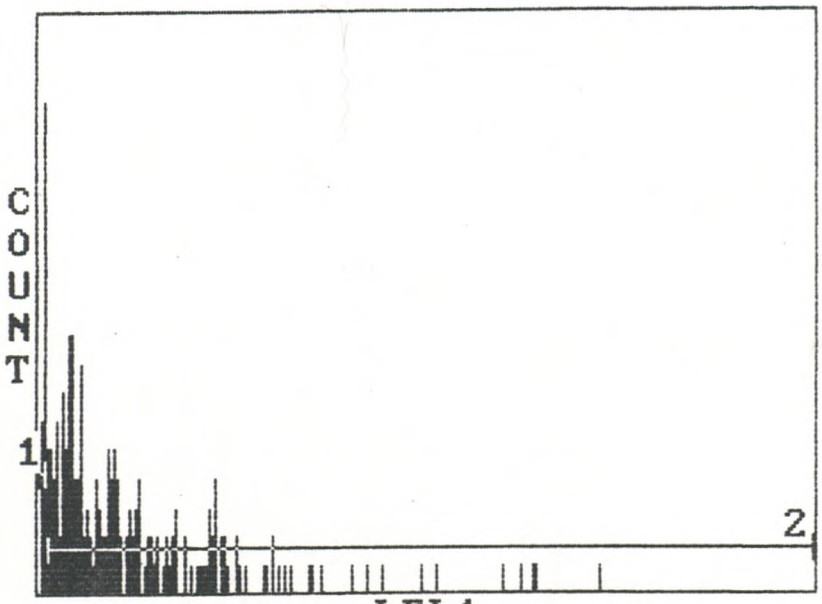


Fig. 33. Prussian Blue stain of blood containing Pappenheimer bodies.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.110	19298	96.5	1.039	1.029	31.7
2	1.110	1023.	702	3.5	2.733	2.771	2.38

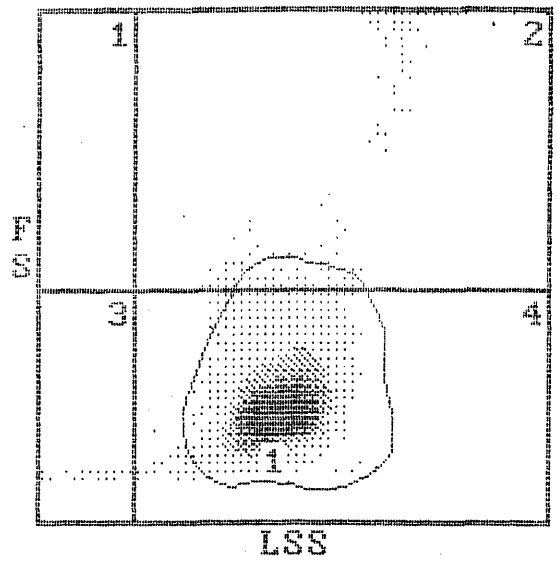
Fig. 34. Histogram from patient with Pappenheimer bodies.

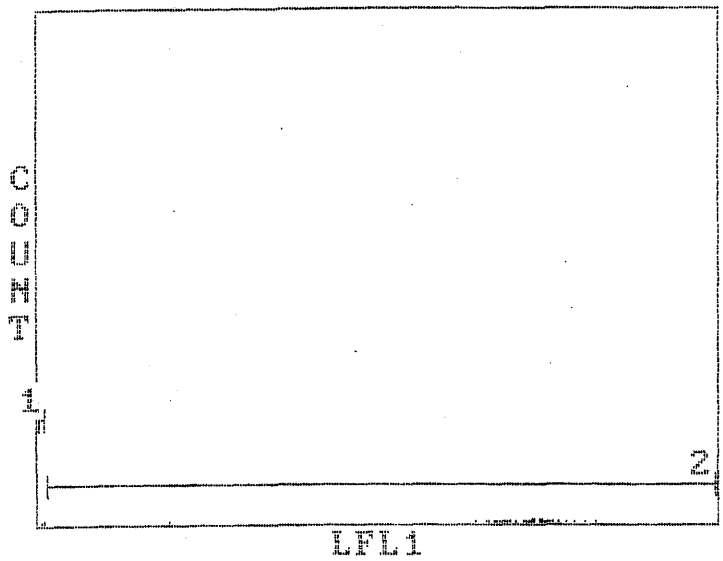
Mature red blood cells are included in the area gated number 1 and the reticulocytes and other fluorescent cells (in this case, cells containing Pappenheimer bodies) are shown in the area gated number 2.

LFL1 = Log of fluorescence

6.7.5 *Chronic Myeloid leukaemia (CML)*

This case demonstrates the advantage of the second histogram to exclude white blood cells from analysis. The manual reticulocyte count on the patient with CML was 0,4%. With the Sysmex R-3000 instrument it was 0,86%. Using the general purpose flow cytometer, it was initially 4,2% (control - 0,2) (Fig. 35A). However, by changing the bitmap on the second histogram to exclude more of the brightly fluorescent cells (Fig. 35B) the reticulocyte count decreased to 1,1%, (Fig. 35C) which is closer to the value obtained by the other two methods.





	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.110	19126	95.6	1.040	1.028	36.0
2	1.110	1023.	874	4.4	57.15	5.61	0.15

Fig. 35A. Histograms from patient with CML.

The first histogram, representing forward scatter (FS) versus log of side scatter (LSS) was used to bitmap the red blood cells. Some white blood cells were situated very close to the red cells, and were therefore included in the bitmap. The second histogram represents the cells included in the bitmap on the first histogram. The reticulocytes and granulocytes are shown in the area gated number 2.

LFL1 = Log of fluorescence.

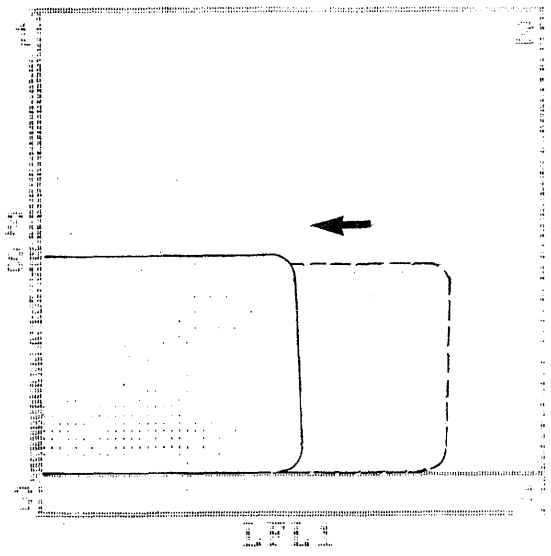
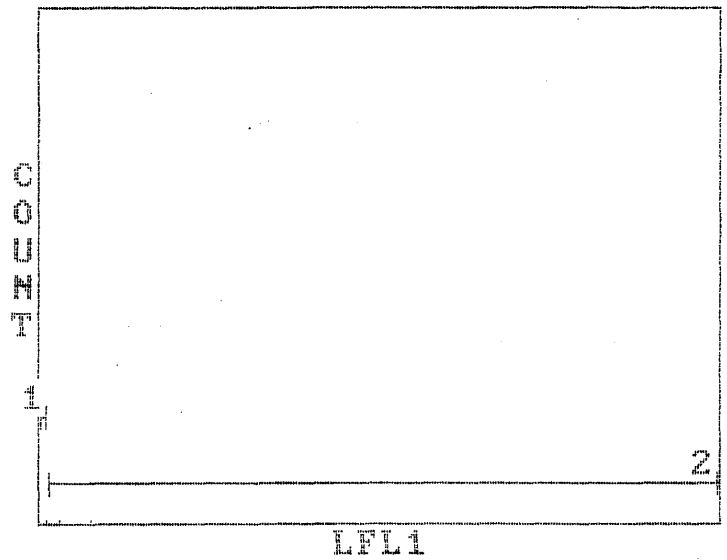


Fig. 35B. Histogram from patient with CML.

On this histogram, representing forward scatter (FS) versus log of fluorescence (LFL1), the bitmap was changed to exclude the most highly fluorescent cells (which includes white blood cells) from analysis.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.110	19742	98.7	1.039	1.028	31.8
2	1.110	1023.	258	1.3	5.287	3.510	1.77

Fig. 35C. Histogram from patient with CML

Only the cells included in the bitmap on the above histogram are represented here. The highly fluorescent cells which were counted as reticulocytes in Fig. 35A are no longer present.

LFL1 = Log of fluorescence.

6.7.6 *Megaloblastic Anaemia*

Using a general purpose flow cytometer to monitor a patient with a severe megaloblastic anaemia on haematinic therapy, the RMI (MCN) value peaked before the peak in the reticulocyte count. At this stage, the haemoglobin level had not changed significantly (Fig. 36) (Controls were all $\leq$ 0,2, and are not shown). The histograms show a low reticulocyte count initially (Fig. 37A), with a peak of immature (highly fluorescent) reticulocytes after 5 days (Fig 37B). The population then shifts (less highly fluorescent cells) as these cells start to mature (Fig. 37C). After a few more days the reticulocyte response starts to decrease (Fig. 37D).

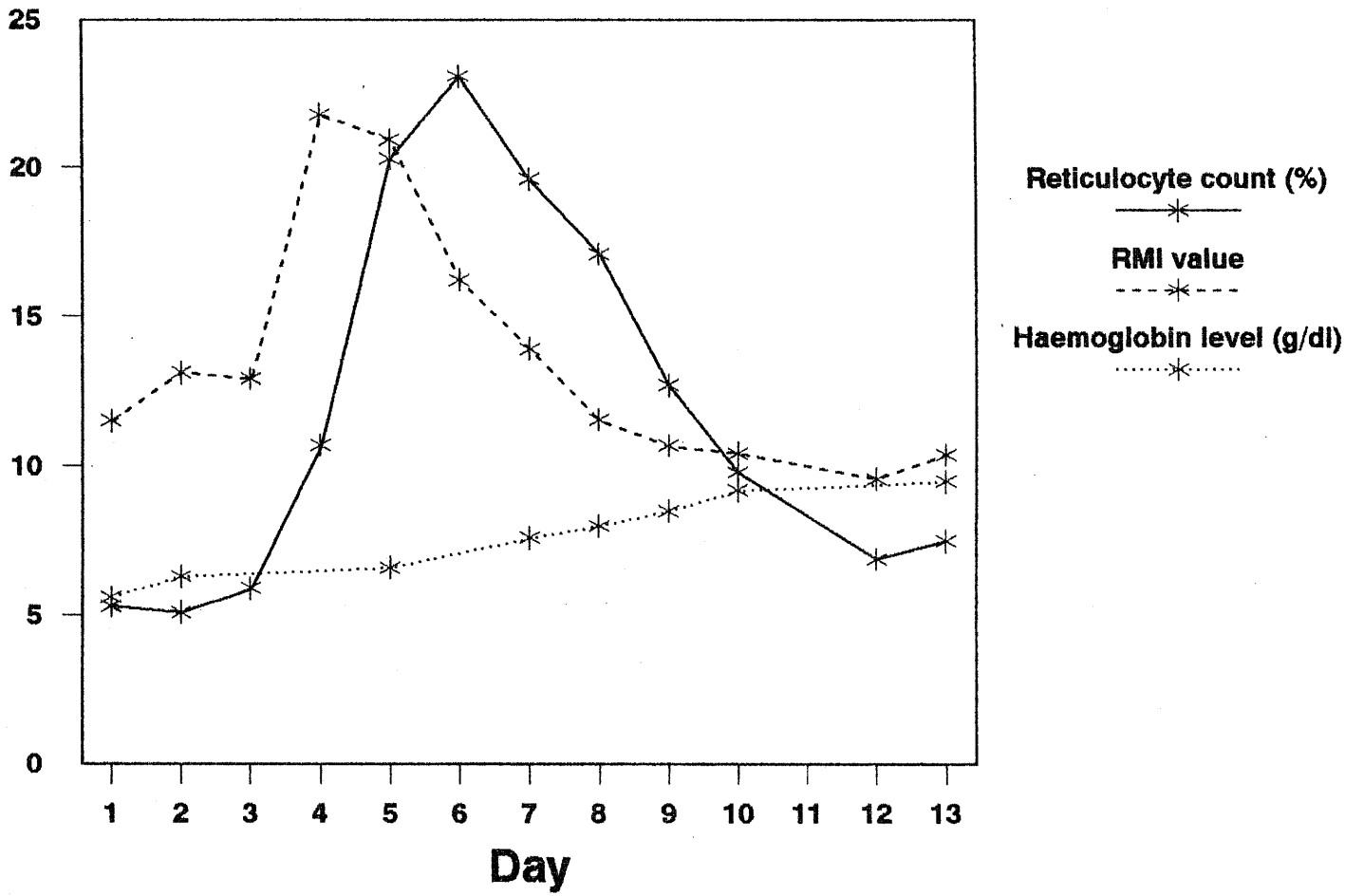
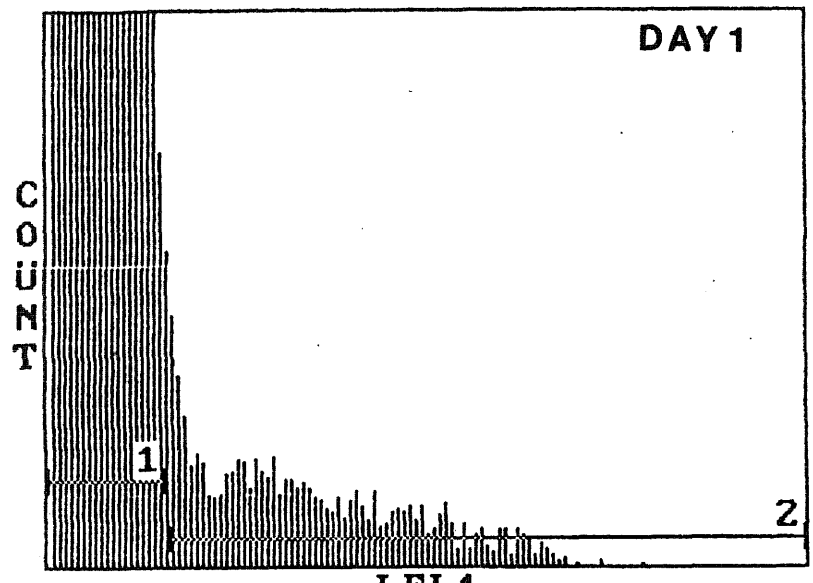


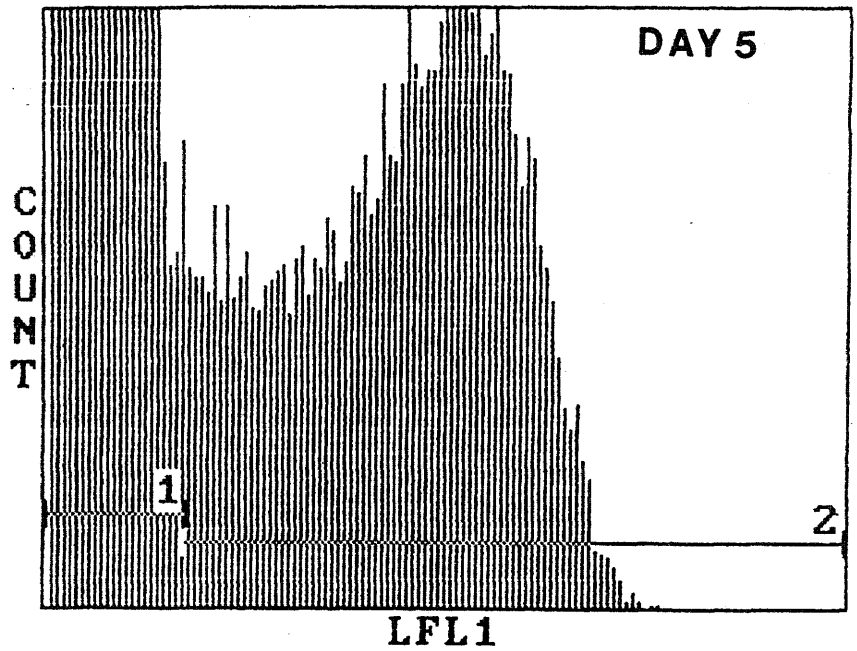
Fig.36. Megaloblastic anaemia on haematinic therapy.

A



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	3.011	29052	94.7	1.581	1.322	42.5
2	3.096	1023.	1618	5.3	11.51	3.09	1.04

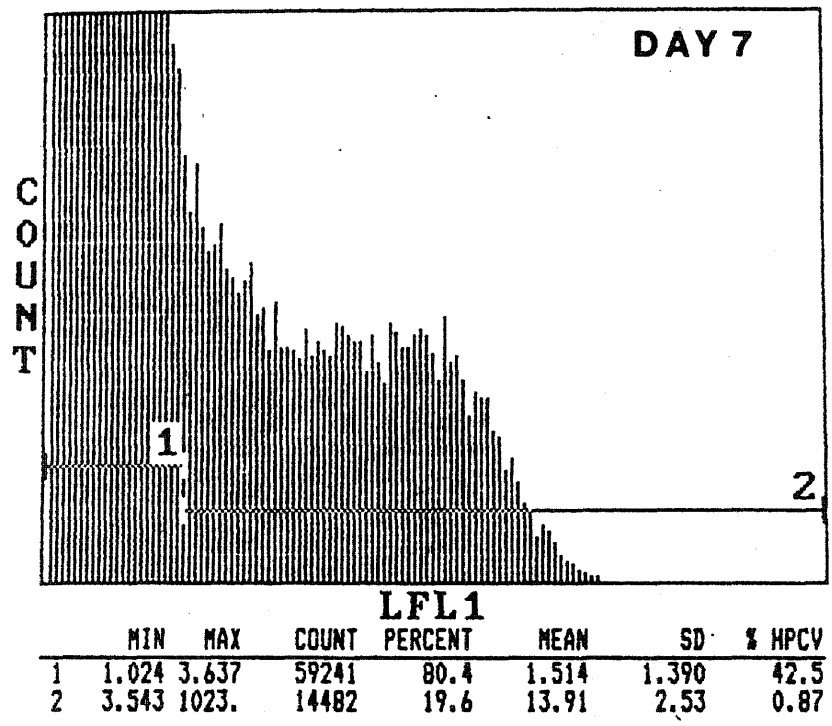
B



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	3.541	35681	80.0	1.473	1.340	42.5
2	3.357	1023.	9059	20.3	20.94	2.60	0.33

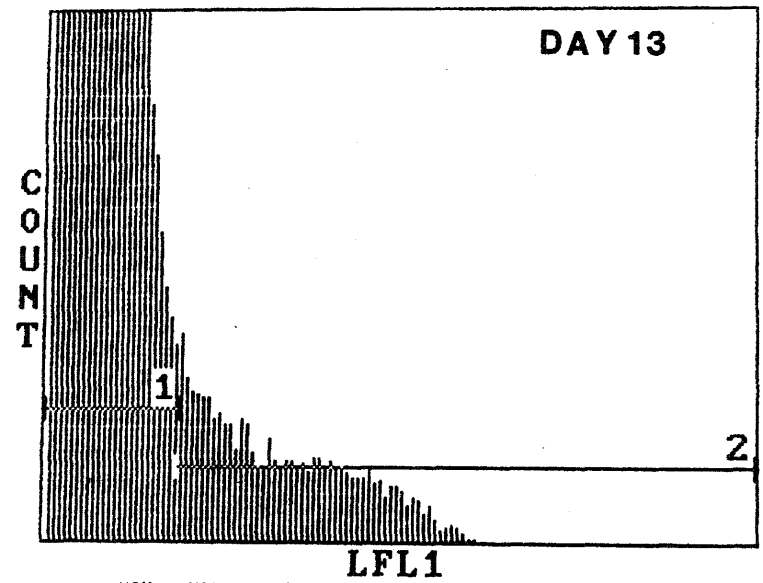
Fig. 37. Histograms from patient with megaloblastic anaemia on haematinic therapy.

C



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	3.637	59241	80.4	1.514	1.390	42.5
2	3.543	1023.	14482	19.6	13.91	2.53	0.87

D



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	3.944	57907	92.9	1.555	1.380	42.5
2	3.739	1023.	4655	7.5	10.40	2.23	0.87

Fig. 37. Histograms from patient with megolablastic anaemia on haematinic therapy.

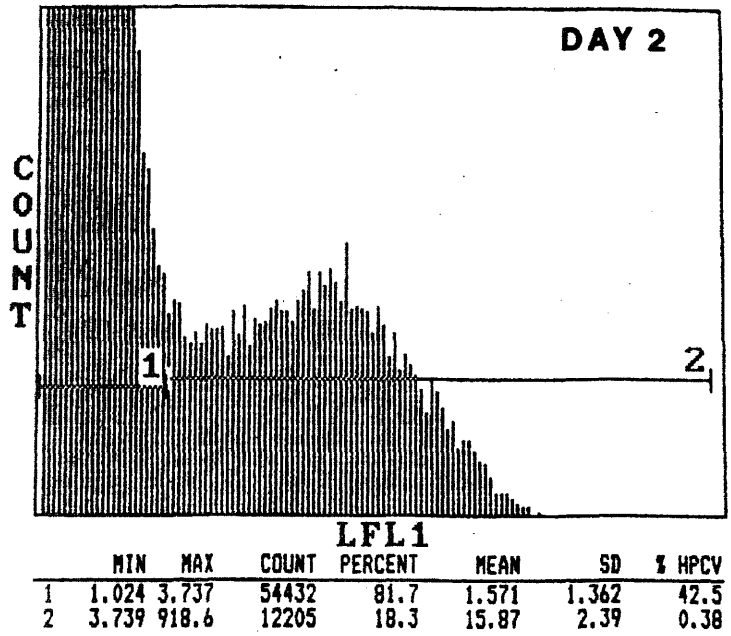
Reticulocytes are represented in area gated number 2. Mature red blood cells are shown in area gated number 1.

LFL1 = Log of fluorescence.

6.7.7 *Haemolytic Anaemia*

The patient with a haemolytic anaemia initially had a high reticulocyte count with a high MCN value of 15,87 (Fig. 38A). As the patient responded to therapy, the reticulocyte count dropped and the RMI value decreased to 8,94 (Fig. 38B). From the histograms, one can see the peak of immature highly fluorescent cells initally. These cells then disappear as the stimulus for erythropoiesis is lost and no new reticulocytes are produced. The immature reticulocytes initially present have matured into old (low fluorescent) reticulocytes.

A



B

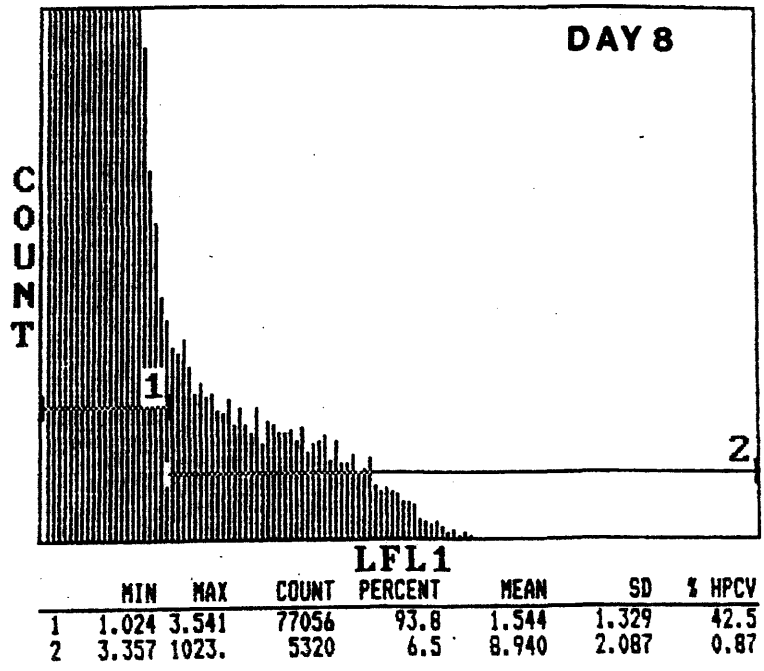


Fig. 38. Histograms of patient with a haemolytic anaemia.

Reticulocytes are represented in area gated number 2 and mature red blood cells in area gated number 1.

LFL1 = Log of fluorescence.

CHAPTER 7

DISCUSSION AND CONCLUSIONS

7.1 **Discussion**

When comparing the three different methods of reticulocyte analysis, they correlated well with one another. This is in agreement with the findings of previous studies. (Lee *et al.*, 1986; Carter *et al.*, 1989; Kojima *et al.*, 1989; Nobes *et al.*, 1990; Van Hove *et al*, 1990; Bowen *et al.*, 1991; Chin-Yee *et al.*, 1991).

The major problem with counting reticulocytes is the fact that there is no clear cut-off point between a reticulocyte and a mature red blood cell, based on the RNA content of the cell. Therefore reticulocytes do not constitute a well defined population. This factor has largely contributed to the difficulties in analysing reticulocytes, both by manual microscopic and flow cytometric techniques. Further studies are needed in order to address this problem.

In this study, a temporary reference material was prepared, by diluting a blood sample with a high reticulocyte count with a sample with a low reticulocyte count, using different concentrations of each. The reticulocyte results obtained using the general purpose flow cytometer correlated exceptionally well with the reference method proposed by this study. The reticulocyte counts obtained manually also correlated very well with the reference method, however, these counts were performed by a very experienced technologist, and so this factor must be taken into account; possibly, when performed by a junior technologist, the results would not be as good. The results obtained using the Sysmex R-3000 instrument were not very good when compared with the reference method. This is most likely related to the fact that this instrument excludes the most highly fluorescent reticulocytes from analysis, as it considers them to be nucleated cells. In fact, the instrument is well known to be inaccurate when analysing reticulocyte counts of > 15% (Sysmex operators manual, R-3000, Kobe, Japan).

When assessing the precision of reticulocyte analysis, both flow cytometric methods were found to have less variation than the intraobserver variation using the manual microscopic method, at all reticulocyte levels. Interobserver variation with manual reticulocyte counting has previously been reported to be even greater than intraobserver variation (Peebles *et al.,* 1981). The precision of the general purpose flow cytometer at low reticulocyte counts however, is not very good (although still better than that of the manual method). This is most likely related to the limitations in distinguishing reticulocytes from mature red blood cells, based on the gates set using the control sample. At higher reticulocyte counts, the precision of the general purpose flow cytometer was actually better than that of the Sysmex R-3000 instrument.

From the above, it appears that the general purpose flow cytometer is better at higher reticulocyte levels, whereas the Sysmex R-3000 is more accurate at low reticulocyte counts. So, the future reticulocyte counters should include the principle used by the Sysmex instruments to discriminate between reticulocytes and mature red blood cells as well as the principle used with the general purpose flow cytometer to exclude nucleated cells from analysis. With all these factors taken into account, flow cytometric reticulocyte analysis has the potential to be very accurate and precise.

Reticulocyte counting using a general purpose flow cytometer would be cost-effective in a clinical laboratory if the flow cytometer was being used for other investigations eg, leukaemia subtyping, T-cell subsets.

A thiazole orange concentration of 1:10 000 was found to give the best results, using the RGP. This is in agreement with the concentration which has been used in previous studies (Lee *et al.,* 1986; Chin-Yee *et al.,* 1991).

The fact that samples can be stored at 4^{O}C for at least 48-72 hours prior to analysis with the RGP (see chapter 3) allows for the batching of samples which is an obvious advantage in a clinical laboratory. The timing of analysis is not critical as specimens can be incubated for 1 to 6 hours prior to analysis with no significant change in the reticulocyte or RMI values, as shown in this study. Lee *et al.* (1986) and Van Hove *et al.* (1990) reported consistent results, with no significant change in the reticulocyte count with incubation times of 30 to 90 minutes and 2 to 7 hours respectively. However, Davis and Bigelow (1989) showed a significant increase in the RMI value after three hours of incubation. A manual reticulocyte count takes $\pm$ 5 mins to perform, whereas with the general purpose flow cytometer each test takes $\pm$ 3 mins to perform (specimen preparation time included). With the Sysmex R-3000 reticulocyte counter, samples can also be kept at 4^{O}C prior to analysis, for up to five days (see chapter 3) and therefore can also be batched. Very little operator time is needed with this instrument as it is fully automated. This instrument would be cost-effective in a laboratory which performs many reticulocyte counts daily (at least 50).

A further advantage of the flow cytometric methods of reticulocyte counting is the RMI estimation, which gives an indication of the age of the reticulocytes in the peripheral blood circulation. This measurement was shown to be useful when monitoring patients with a haemolytic anaemia, or a megaloblastic anaemia on haematinic therapy. Davis and Bigelow (1990) and Cavill (1990) showed the advantage of the RMI measurement in patients with a megaloblastic anaemia on haematinic therapy.

The MCN and HFR values are measuring different variables. Although they were shown to correlate in a previous study (Bowen *et al.*, 1991), the present study showed a very poor correlation between these two measurements.

Both flow cytometric instruments could not discriminate between malaria parasitized red blood cells and reticulocytes. Laharrague *et al.* (1990) and Coulet and Bezou (1990) also found false reticulocyte results in the presence of malaria parasites, using the Sysmex R-1000 instrument. However, the presence of a peak of fluorescent cells in the lower fluorescence channels only should alert the observer to analyse the specimen manually, as this is a very unusual picture for a reticulocytosis.

Care should be taken when performing flow cytometric reticulocyte counts on patients with markedly elevated white blood cell counts. Problems in interpretation in patients with CLL have been reported previously (Chin-Yee *et al.*, 1991). The Sysmex R-3000 instrument excludes nucleated cells (WBC's) from analysis, based on their fluorescence intensity. With the general purpose flow flow cytometer, white blood cells are excluded with bitmapping facilities, based on their different size, internal content and fluorescence intensity. However, it is advisable that specimens with very high white cell counts be checked manually, especially when using a general purpose flow cytometer.

Sickled red cells did not cause problems with flow cytometric reticulocyte analysis in this study, which is in contrast with another report which claimed that sickled red cells are excluded from analysis (Ferguson *et al.*, 1990).

Pappenheimer bodies were counted as reticulocytes by the general purpose flow cytometer, whereas this problem did not occur with the Sysmex R-3000 instrument as previously reported (Oyamatsu *et al.*, 1989). This is most likely related to the different dyes used with the two methods.

7.2 Conclusions

Although the overall cost of reticulocyte analysis by flow cytometric methods is higher than with the manual microscopic method, based on the cost of reagents, the improved precision and accuracy as well as the RMI estimation with the flow cytometric methods make this technique obviously advantageous.

A reticulocyte count should become included as an additional independent parameter on new generation three - or five - part differential analyses. This would obviously be cost-effective as it would eliminate the expense of buying an instrument which is dedicated to counting only reticulocytes.

The biggest problem with the flow cytometric reticulocyte counters is that there is no accepted reference method with which to compare them (see chapter 3). A reference method needs to be developed before the full value of flow cytometric reticulocyte counting can be accurately assessed. The method used in this study to make a reference material may be useful for future investigations.

The RMI value has been shown to be an independent measurement, which has clinical utility. However, the units of measurement must be standardized and normal ranges determined before it can be recognized and used as a standard laboratory parameter.

With flow cytometric methods, the measurement of reticulocytes can now be used as a sensitive index of bone marrow erythropoietic output, whereas previously, with the manual microscopic method, it could only detect gross changes in erythropoiesis. This is of obvious benefit to clinicians, who should be educated as to the advantages of this technique, so that it can be utilized appropriately to gain the maximum benefit.

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APPENDIX

Incubation

Table 5. Effect of different incubation times on reticulocyte results

Time	Specimens							
Hours	1	2	3	4	5	6	Two-Tail P-Value*	
0.25	1.5	1.1	2.4	2.2	1.7	7.6		
0.5	1.6	1.2	3.4	2.8	1.5	9.6	} 0.1563	**0.0313**
1	1.9	1.3	3.8	3.3	1.5	10.2	} 0.0625	
1.5	2.0	1.5	4.0	3.5	1.9	10.2	} 0.0625	
2	1.9	1.3	4.3	3.6	1.6	9.5	} 0.3125	
3	1.9	1.6	4.0	3.9	2.1	9.6	} 0.1875	
4	1.9	1.5	4.1	3.9	2.3	9.2	} 1.0000	
5	1.5	1.6	4.2	3.4	2.2	8.1	} 0.2500	
6	1.1	1.4	4.8	2.9	1.5	7.9	} 0.2813	
24	2.6	3.0	6.1	4.6	3.7	10.6	} **0.0313**	

* Results of Wilcoxon test, to determine whether reticulocyte results at different incubation times are significantly different from one another or not. (> 0,05 = not significantly different; < 0,05 = significantly different).

Table 6. Effect of different incubation times on MCN value

Time	Specimens							
Hours	1	2	3	4	5	6	Two-Tail P-Value*	
0.25	4.055	3.399	3.432	3.012	3.375	2.888		
0.5	4.613	3.603	4.486	3.473	3.305	3.212	} 0.0625	} **0.0313**
1	5.190	4.083	5.333	4.047	3.481	3.728	} **0.0313**	
1.5	5.474	4.358	5.632	4.687	3.422	4.222	} 0.0625	
2	5.121	4.139	5.847	4.441	3.460	4.227	} 0.4375	
3	4.640	3.987	6.135	4.455	3.150	4.546	} 0.8438	
4	4.960	3.485	5.769	4.389	3.228	4.227	} 0.4375	
5	4.678	3.905	5.760	3.921	3.153	4.488	} 0.6875	
6	4.543	4.042	5.826	4.099	3.545	4.736	} 0.0938	
24	6.406	5.502	6.989	5.737	5.114	4.807	} **0.0313**	

* Results of Wilcoxon test, to determine whether the mean channel number (MCN) results at different incubation times are significantly different from one another or not.
(> 0,05 = not significantly different; < 0,05 = significantly different).

Accuracy

Table 7. Weights of Distilled Water Pippetted

	0.9ml	**0.75ml**	**0.25ml**
	0.884g	0.734g	0.228g
	0.882g	0.732g	0.234g
	0.876g	0.728g	0.212g
	0.879g	0.725g	0.228g
	0.868g	0.730g	0.229g
	0.868g	0.737g	0.235g
	0.866g	0.722g	0.212g
	0.844g	0.715g	0.228g
	0.844g	0.722g	0.225g
	0.852g	0.718g	0.220g
mean	**0.8663**	**0.7263**	**0.2251**
SD	**0.0150**	**0.0071**	**0.0081**
CV =	**1.73%**	**0.98%**	**3.60%**

	0.99ml	**0.01ml**	**0.1ml**
	0.940g	0.010g	0.091g
	0.940g	0.010g	0.093g
	0.939g	0.012g	0.092g
	0.941g	0.010g	0.093g
	0.936g	0.010g	0.092g
	0.940g	0.011g	0.090g
	0.941g	0.010g	0.092g
	0.939g	0.010g	0.095g
	0.940g	0.010g	0.094g
	0.939g	0.011g	0.094g
mean	**0.9395**	**0.0104**	**0.0926**
SD	**0.0014**	**0.0007**	**0.0015**
CV =	**0.15%**	**6.73%**	**1.62%**

Table 8. *Dilutions (ml) of blood samples*

	High reticulocyte count specimen	*Low reticulocyte count specimen*
1	1.0	-
2	0.7263	0.7263
3	0.2251	0.7263
4	0.0926	0.8863
5	0.0104	0.9395
6	-	1.0

Table 9. *Results of reticulocyte analysis by RM, RGP, RD and the reference values.* (Specimens 1-6 based on above dilutions)

	RGP PMT				Manual	R-3000	Reference
Specimens	900	950	975	1050			
1	31.1	36.0	39.7	38.6	40.6	29.06	37.94
2	17.7	20.2	21.5	21.9	23.2	24.97	19.23
3	10.0	11.0	11.3	11.4	10.8	14.08	9.37
4	4.5	5.4	5.3	5.7	4.6	6.12	4.12
5	1.5	2.1	1.8	1.7	0.4	0.76	0.92
6	0.9	1.3	1.2	1.4	0.4	0.35	0.51

Precision

Table 10. Precision of reticulocyte counting by RM, RGP and RD

	RGP	RD
High reticulocyte count	5.1 4.8 5.0 4.6 4.6 4.3 4.9 4.8 4.5 4.5	5.40 5.15 5.23 5.22 5.18 5.40 5.11 6.21 5.03 4.50
mean **SD** **CV =**	**4.710** **0.2514** **5.34%**	**5.2430** **0.4233** **8.07%**
Intermediate reticulocyte count	1.0 1.1 1.1 1.0 1.1 1.0 1.0 1.1 1.0 1.0	1.06 1.19 1.22 1.36 1.27 1.11 1.34 1.12 1.28 1.21
mean **SD** **CV =**	**1.040** **0.0516** **4.96%**	**1.2160** **0.0992** **8.16%**
Low reticulocyte count	0.1 0.2 0.2 0.1 0.1 0.2 0.1 0.1 0.2 0.2	0.27 0.30 0.37 0.25 0.29 0.31 0.35 0.27 0.32 0.33
mean **SD** **CV =**	**0150** **0.0527** **35.13%**	**0.3060** **0.0378** **12.35%**

Table 10 contd.	*RM*				
	Observers				
	1	2	3	4	
Low reticulocyte count	0.2	0.2	0.4	0.2	
	0.2	0.4	0.4	0.8	
	0.2	0.2	0.4	0.2	
	0.4	0.4	0.2	1.0	
	0.6	0.4	0.2	0.2	
	0.2	0.2	0.4	0.2	
	0.2	0.2	0.2	0.2	
	0.4	0.2	0.4	0.6	
	0.2	0.2	0.2	1.0	
	0.2	0.2	0.4	0.4	
mean	**0.280**	**0.260**	**0.3200**	**0.480**	
SD	**0.1398**	**0.0966**	**0.1033**	**0.3425**	
CV =	**49.93%**	**37.15%**	**32.28%**	**71.35%**	**Average** 47.68%
Intermediate reticulocyte count	2.0	3.0	2.6	1.8	
	2.0	3.0	1.8	3.0	
	2.2	2.0	2.4	2.2	
	1.6	2.6	1.4	2.8	
	2.6	2.4	1.6	3.0	
	2.2	2.8	2.0	2.6	
	2.2	2.6	1.2	1.6	
	2.0	2.8	2.4	2.0	
	1.4	2.2	2.4	2.0	
	1.8	2.6	1.8	2.0	
mean	**2.0**	**2.6**	**1.960**	**2.3**	
SD	**0.3399**	**0.32696**	**0.4789**	**0.5099**	
CV =	**17%**	**12.56%**	**24.43%**	22.17%	**Average** 19.04%
High reticulocyte count	10.6	7.6	6.0	8.4	
	10.2	6.6	5.6	9.1	
	9.0	8.0	7.4	7.4	
	9.6	6.6	7.0	11.6	
	9.8	8.0	6.4	10.2	
	11.0	7.8	6.0	8.8	
	10.2	7.2	8.0	11.8	
	10.2	7.4	6.6	9.6	
	10.0	6.4	5.6	7.6	
	9.8	7.2	5.2	7.6	
mean	**10.04**	**7.280**	**6.380**	**9.21**	
SD	**0.5481**	**0.5903**	**0.8817**	**1.5934**	
CV =	**5.46**	**8.11%**	**13.82%**	**17.30%**	**Average** 11.17%

Correlation between RM, RGP and RD.

Table 11. Results of reticulocyte testing by RM, RGP and RD

Specimens	RGP PMT 950	RGP PMT 975	RGP PMT 1000	RM	RD
1	2.1	2.5	2.9	3.5	1.54
2	2.3	2.5	3.2	2.0	3.01
3	2.5	2.6	2.9	4.4	2.55
4	1.9	2.2	2.3	2.8	1.67
5	0.8	1.0	1.0	1.3	0.94
6	0.3	0.2	0.4	0.2	0.16
7	1.6	1.9	2.2	2.1	1.97
8	13.8	14.6	16.9	17.8	16.28
9	5.4	6.2	6.4	13.3	7.54
10	16.9	17.0	17.5	15.8	13.54
11	5.6	5.7	6.4	8.6	6.85
12	1.9	2.7	3.1	2.3	1.61
13	5.1	6.1	6.0	7.8	7.23
14	2.1	2.3	2.7	4.6	2.02
15	2.1	2.8	2.9	2.7	2.06
16	1.5	1.9	2.0	0.8	1.17
17	11.0	11.5	11.9	13.9	10.39
18	1.3	1.8	2.1	1.2	0.84
19	0.8	1.7	2.0	1.2	1.27
20	0.3	0.7	0.9	0.2	0.46
21	0.7	0.8	1.0	0.2	0.71
22	0.6	0.8	0.7	0.4	0.52
23	0.3	0.4	0.7	0.2	0.14
24	13.8	15.5	17.1	16.2	8.35
25	10.3	10.8	10.7	18.3	11.52
26	0.9	1.0	1.2	0.5	0.85
27	0.5	0.5	0.7	0.2	0.59
28	0.4	0.5	0.4	0.2	0.35
29	22.6	24.4	26.7	25.1	27.69
30	0.1	0.3	0.3	0.4	0.27
31	1.0	1.2	1.3	2.2	1.06
32	0.3	0.3		0.2	0.41
33	0.5	0.8		0.2	0.64
34	0.7	0.6		1.2	0.85
35	0.3	0.3		0.4	0.64
36	0.0	0.0		0.2	0.05
37	0.2	0.2		0.2	0.17
38	1.1	1.3		0.6	1.52
39	0.3	0.4		0.2	0.41

Comparison of MCN and HFR

Table 12. Comparison of MCN and HFR measurements

Specimen	MCN	HFR %
1	2.588	3.2
2	2.966	1.0
3	3.753	2.0
4	2.824	0.0
5	3.670	0.0
6	4.788	5.7
7	7.995	2.6
8	3.083	0.0
9	4.579	0.4
10	6.666	5.2
11	6.184	4.7
12	5.990	26.9
13	6.785	15.2
14	6.080	0.0
15	3.487	0.0
16	3.043	0.9
17	3.759	5.2
18	6.040	19.3
19	4.789	1.6
20	4.140	5.4
21	4.512	1.3
22	4.453	2.5
23	3.873	0.3
24	9.602	3.8
25	4.236	0.8
26	4.165	11.5
27	4.354	19.5
28	7.228	2.6
29	5.232	11.0
30	4.742	6.5
31	5.106	10.5
32	5.517	5.8
33	3.857	3.5
34	4.206	4.4
35	9.027	4.6
36	6.149	5.8

Table 12 contd.

Specimen	MCN	HFR %
37	3.062	0.8
38	5.208	5.7
39	6.341	10.3
40	4.797	2.4
41	3.766	0.3
42	3.984	4.5
43	4.548	5.5
44	6.486	6.1
45	4.589	2.7
46	4.243	2.4
47	4.590	2.5
48	7.353	10.0
49	5.163	4.0
50	3.492	0.0
51	5.746	3.3
52	3.197	3.4
53	3.132	0.0
54	3.234	0.6
55	4.106	15.3
56	3.175	1.7
57	6.129	11.4
58	4.372	1.7
59	5.479	6.2
60	6.914	6.0
61	4.660	2.4
62	5.128	5.4
63	3.961	3.8
64	5.803	6.3
65	4.861	3.7