CHAPTER 18

Flow Cytometry in Malaria Detection

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I. Introduction

Malaria is a parasitic disease found in tropical and subtropical regions which is caused by unicellular organisms of the genus *Plasmodium*. In man, 4 different species are responsible for the disease, *Plasmodium falciparum, P. vivax, P. ovale,* and *P. malariae*.

A large part of the life cycle of these parasites takes place in the blood circulation, where these organisms invade red blood cells in which they grow and multiply. Failure of existing methods to control malaria, the lack of an effective vaccine, and increasing drug resistance of the parasites are factors which play a role in the increase of malaria cases. Both for laboratory research aimed at the development of new control strategies and for monitoring the effects of existing control projects in the field, the availability of rapid, sensitive, and reproducible techniques for the detection and analysis of blood infection are required. The blood stage infection is the most relevant part of the life cycle; the blood stages cause the clinical symptoms and are targets for a number of drugs. Moreover, the demonstration of the presence of parasites in the blood is used for diagnosis and treatment of the disease.

Flow cytometry has proven to be a useful tool for the analysis of blood infection by malaria parasites. Analysis of blood-stage development (Janse *et al.*, 1987; Mons and Janse, 1992) and determination of susceptibility to drugs by flow cytometry (van Vianen *et al.*, 1990a,b) are reproducible and rapid and detection of blood-stage parasites appears to be sensitive and reproducible (van Vianen *et al.*, 1993; P. H. van Vianen, unpublished results).

The analysis and detection of malaria infection by flow cytometry makes almost exclusive use of fluorescent dyes which are specific for nucleic acids (for review, see Mons and Janse, 1992). DNA-specific dyes are especially useful since the parasites multiply inside the red blood cell (RBC) population of the blood cells. Since RBC do not contain DNA, DNA-specific fluorescence from infected RBC can only be due to fluorescence of dyes bound to parasite DNA. Consequently, infected cells can be discriminated from noninfected cells based on their fluorescence intensity. In addition, since parasites multiply within the RBC by several mitotic divisions, the fluorescence intensity of stained parasites

For flow cytometry the blood stages (see Fig. 1) are the most important (see below). After entering the RBC the small haploid parasites (called ring forms) develop and grow until they nearly fill the RBC (these growing stages are the so-called trophozoites). After this growth phase most of the parasites synthesize DNA and enter mitosis. In each parasite, now called schizont, three to five rapid mitotic divisions follow each other, resulting in the production of 8–32 haploid merozoites. After rupture of the RBC, these merozoites can penetrate new RBC and the multiplication cycle starts again. One multiplication cycle takes 24–72 hr, depending on the species. This mitotic multiplication results in a rapid increase in the number of infected red blood cells.

A small percentage of the merozoites do not continue the asexual multiplication but differentiate within the RBC into precursor cells of the gametes, the so-called gametocytes. These stages develop into gametes when they are ingested by mosquitoes, in which further development takes place. No flow cytometric studies on the mosquito stages of the parasite have been reported.

B. Flow Cytometry and Determination of Parasite Development, DNA Synthesis, and Drug Susceptibility

The blood stages of several species of malaria parasites can grow and multiply under culture conditions. These *in vitro* cultures allow the effect of new drugs to be studied on parasite development and DNA synthesis under standardized

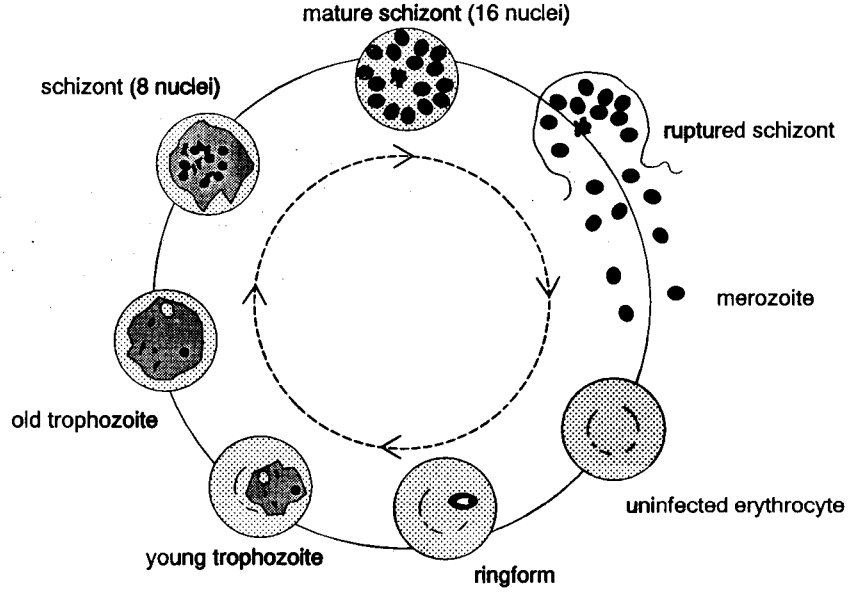


Fig. 1 Schematic representation of blood-stage development of malaria parasites. Only part of the life cycle, the asexual erythrocytic development, is shown.

increases during development of the parasites. This can be analyzed by flow cytometry and used to determine the developmental stage of the parasite. The total DNA content of a parasite is 100–200 times less than that of nucleated blood cells. Therefore, the nucleated blood cells can easily be distinguished from parasites on the basis of the difference in fluorescence intensity.

The vast majority of the reported flow cytometric studies on malaria parasites have been carried out with the A/T-specific DNA dyes, Hoechst 33258 and Hoechst 33342. These dyes give a strong fluorescence with parasite DNA after fixation of infected blood cells (Myler *et al.*, 1982; Bianco *et al.*, 1986) or free parasites and after vital staining of parasites (Franklin *et al.*, 1986). Moreover, the relative fluorescence intensity of different blood stages after Hoechst staining corresponds closely to the relative DNA content of these stages (Janse *et al.*, 1987). A/T-specific dyes are particularly suited since the A/T content of DNA of malaria parasites is extremely high, ranging from 70 to 82% in different species. These dyes are now routinely used in studies to determine parasite development and DNA synthesis in parasites and for determination of the level of drug resistance in parasites obtained from patients. Moreover, it has been shown that these dyes can be used for sensitive detection of parasites in blood samples obtained from patients in clinical and in epidemiological studies (van Vianen *et al.*, 1993; P. H. van Vianen, unpublished results).

Other dyes have been used for parasite detection (for review, see Mons and Janse, 1992), such as acridine orange (Hare, 1986; Whaun *et al.*, 1983), propidium iodide (Saul *et al.*, 1982; Pattanapanyasat *et al.*, 1992), and thiazole orange (Makler *et al.*, 1987). These dyes have the disadvantage that they bind both to DNA and to RNA. The reticulocyte population of blood cells contains RNA and the fluorescence of parasite-infected cells can be in the same range as that of noninfected reticulocytes which hampers the discrimination between infected cells and noninfected cells.

Below we describe flow cytometric methods for (1) measurement of parasite development and DNA synthesis by parasites, (2) determination of susceptibility of parasites to drugs, and (3) detection of low numbers of parasite-infected cells in blood samples from patients.

II. Applications

A. Flow Cytometry and the Developmental Cycle of the Parasite

Malaria parasites have a complex life cycle which alternates between two different hosts: mosquitoes and vertebrates such as reptiles, birds, rodents, nonhuman primates, and humans. An infection starts with a bite of an infected mosquito, which injects parasites into the blood of the vertebrate host. These parasites penetrate liver cells and, after one developmental cycle, the parasites are released in the blood and invade RBC.

conditions. The effect of new drugs on parasite development in culture is routinely monitored by microscopic counting of the number of infected cells or by measuring the incorporation of radioactive precursors into the nucleic acids of the parasites. Flow cytometry is a very good alternative for the determination of the effect of new drugs on parasite development in culture. Using DNA-specific dyes the increase/decrease of the number of infected cells can be measured rapidly and reproducibly. In addition, the amount of DNA synthesis can be determined precisely which is a reliable characteristic of the development of the parasites (Janse *et al.*, 1987,1989; van Vianen *et al.*, 1990a). The advantages of flow cytometry are the speed of measurement, the accuracy, reproducibility, and the large number of parasites analyzed.

For the determination of drug susceptibility of parasites from field isolates the World Health Organization (WHO) (1982) has developed a standard *in vitro* microtest. In these tests haploid small blood stages (ring forms, trophozoites) are cultured for short periods in the presence of different concentrations of drugs. Giemsa-stained slides are made from these cultures to monitor development of the parasites from ring forms into the DNA synthesizing stages (schizonts) by light microscopy. Determination of parasite development by light microscopy is time consuming and the results can easily be influenced by human errors. Since development and DNA synthesis of parasites can accurately be assessed by flow cytometry using DNA-specific dyes, flow cytometry is therefore very useful for determination of drug susceptibility. Recently, a method has been developed for the fully automated reading of the WHO microtests by flow cytometry (van Vianen *et al.*, 1990b).

C. Flow Cytometry and Detection of Blood Stages

The demonstration of the presence of blood stages is used for diagnosis and treatment of malaria. The "gold standard" is the microscopic detection of blood stages in thin or thick blood smears which are stained with Giemsa. However, sensitive detection in large numbers of blood samples collected from patients under primitive field conditions using this method poses problems. The sensitivity is highly influenced by local circumstances and working conditions and depends on the availability of experienced people for recognition of parasites. The quality of microscopic slides is not always optimal, due to improper preparation of the smears, applying contaminated staining solution, or suboptimal use of the staining procedure. The sensitivity is very much dependent on the experience of the microscopist and the time spent reading the slides. Because microscopy is labor intensive, human factors such as loss of concentration, especially when large numbers of samples need to be screened with a low percentage of positives, can account for misreading of samples (Payne, 1988).

In the past few years, alternatives to microscopic detection of malaria parasites have been investigated, such as immunological methods to demonstrate antibodies or antigens (Tharavanij, 1990), detection of parasites by fluoresence

microscopy (Rickman *et al.*, 1989; Kawamoto and Billingsley, 1992), and the use of malaria-specific radioactively labeled DNA and RNA probes (Tharavanij, 1990). At present none of these techniques appears to be superior to microscopic examination of Giemsa-stained blood smears. Flow cytometry has been shown to be potentially suited to overcome most of the above-mentioned problems with parasite detection (van Vianen *et al.*, 1993). Although several fluorescent dyes have been reported to be useful for detection of parasite-infected cells most studies have been performed with the DNA-specific Hoechst dyes (Mons and Janse, 1992). Detection and counting of the number of infected cells can be performed using Hoechst 33258-stained RBC which are fixed by glutaraldehyde. However, this method is not sensitive enough to detect very low numbers of infected RBC (<0.1%). It appears to be necessary to first free the parasites from the RBC by lysing these blood cells (van Vianen *et al.*, 1993). This reduces the sample volume and the number of cells to be analyzed.

III. Materials

Materials for the *in vitro* culture of *P. berghei* have been described by Janse *et al.* (1989). For materials for *in vitro* cultures of *P. falciparum* see references in Trigg (1985).

Phosphate-buffered saline (PBS) tablets (Flow Laboratories) are dissolved in double-distilled deionized (demi) water and HCl is used to adjust the pH at 7.2. PBS for fixation solution or staining solution is filtered through a 0.22-μm filter to remove small particles. PBS is stored at 4°C.

Hoechst 33258 (Janssen Chimica) is dissolved in demi-water at a stock concentration of 500 μM. The stock is stored at −20°C. Final concentration for cell staining is 2 μM in PBS.

Propidium iodide (Sigma) is dissolved in demi-water at a stock concentration of 1 mg/ml, which is stored at 4°C. Final concentration for cell staining is 1 μg/ml in PBS.

Glutaraldehyde (Zeiss, high grade, 70%) is diluted with PBS at a stock concentration of 25% and stored at −20°C. Final solution is made by diluting the stock $\frac{1}{100}$ with filtered PBS (to 0.25%) and stored at 4°C.

Lysis solution (Becton–Dickinson Immunocytometry Systems, 10× stock) is stored at room temperature. Final solution is made by diluting the stock $\frac{1}{10}$ with demi-water and filtering through a 0.22-μm filter. Storage is at 4°C.

IV. Cell Preparation and Staining

A. Collection of Blood Samples from Infected Humans and Laboratory Animals

For flow cytometric analysis only small blood samples are required. Samples from human patients can be taken from blood collection tubes treated with heparin or EDTA as anticoagulants, which are routinely used for collection of

blood. Alternatively small samples can be drawn using heparinized capillaries from the finger after a finger prick.

Malaria parasites which infect nonhuman primates and rodents are regularly used as models for the study of malaria. Small blood samples (20–200 μl) from infected rodents, such as mice and rats, are usually collected from the veins at the end of the tail using heparinized capillaries and resuspended in PBS or culture medium. Cells are collected by centrifugation for 5 sec (15,000*g*) in an Eppendorf centrifuge or at 450*g* for 10 min. When larger amounts of blood are needed (for example for cultures of the blood stages; see below), a cardiac puncture under etheranesthesia is performed and blood is collected either in PBS containing heparin (20 IU/ml) or in culture medium RPMI 1640 (see below) containing heparin (20 IU/ml). Heparin is added as an anticoagulant. Cells are collected by centrifugation at 450*g* for 10 min.

B. Collection of Samples from *in Vitro* Cultures of the Blood Stages of Malaria Parasites

In vitro cultures of the blood stages of two species are regularly used for the study of parasite development and drug susceptibility. These are the human parasite *P. falciparum* and a parasite which infects rodents, *P. berghei*. Culture methods for both species have been described extensively (Trigg, 1985; Janse *et al.*, 1989). Here the methods are described very briefly.

Infected RBC are obtained either directly from humans and rodents or from liquid nitrogen storage. Cultures are normally started with young stages of the parasite, the ring forms. Infected RBC are incubated in culture medium RPMI 1640 containing Hepes buffer (5.94 g/liter) $NaHCO_4$, serum (10–20%), and antibiotics. This cell suspension at an RBC concentration ranging from 0.5 to 10% is incubated at 37°C in culture plates, flasks, petri dishes, or Erlenmeyers and gassed with a mixture of 10% CO_2, 5% O_2, and 85% N_2. In these cultures parasites develop from ring forms into schizonts, after which invasion of new RBC takes place. For both parasite species methods have been described to synchronize the asexual development of the blood stages, so that all parasites are at the same stage of development during the complete cycle. Samples from the culture are centrifuged for 5 sec (15,000*g*) in Eppendorf centrifuges or at 450*g* for 10 min to remove culture medium.

C. Collection of Samples from Standard Drug Susceptibility Tests

For determination of drug susceptibility of *P. falciparum* parasites obtained from patients, the WHO (1982) has developed a standardized microtest. Infected blood, obtained from patients, is incubated in complete culture medium (RPMI 1640 + 10% human serum) in standard 96-well microtiter plates for 26–30 hr, according to the WHO procedure, at an RBC concentration of 10%. The plates are predosed with different concentrations of drugs. Samples from the microtest can be prepared as described for the *in vitro* cultures of the blood stages of the

parasites. However, for flow cytometric analysis the samples can remain in the culture wells at the end of the culture period and be fixed and stained in the wells after removal of the culture medium (see below).

D. Fixation of Infected Erythrocytes

Infected RBC can be fixed with glutaraldehyde, paraformaldehyde, or a combination of these two. Both glutaraldehyde and (para)formaldehyde have the disadvantage that they crosslink cell membrane components, which hampers the penetration of high-molecular-weight molecules such as monoclonal antibodies, DNA probes, or large fluorochromes. In addition these fixatives can have a significant quenching effect on the emission of fluorescence from certain DNA-bound fluorochromes (Crissmann *et al.*, 1979). Despite these disadvantages aldehyde-type fixatives appear to be very useful for fixation and staining of infected RBC with Hoechst dyes. They induce no significant cell aggregation or lysis, which are frequently observed when for example ethanol and methanol are used as fixative.

The usual procedure for fixation is as follows: Samples of infected blood cells obtained from humans, laboratory animals, or cultures are washed once in PBS before fixation. Typically these samples consist of 1 ml of blood cell suspension of 0.5–10% (10^7–10^9 cells), collected in eppendorf tubes. The blood cells are centrifuged for 5 sec at 15,000*g* and the supernatant is removed. Subsequently 1 ml of 0.25% glutaraldehyde in PBS is added and the sample is mixed vigorously. Fixation is done at 4°C for 15 min. The cell suspension in the glutaraldehyde solution ranges between 0.5 and 10%. After fixation, cells are washed twice with PBS. Fixed cells are stored in PBS at 4°C until being stained for flow cytometry. Cells can be kept for more than a year at 4°C without deteriorating. We have found that the washing steps with PBS both before and after fixation are not necessary for accurate flow cytometric readings. Blood cells can be added directly to the fixative and can be stored without removal of the fixation solution.

E. Fixation of Free Parasites

To detect very low numbers of parasites in blood samples from patients it is beneficial to lyse the RBC before fixation in order to reduce the sample volume and the number of cells to be analyzed (van Vianen *et al.*, 1993). For this method samples of 50 μl of blood (1–5 $\times$ 10^8 cells) are collected in eppendorf tubes with 1 ml of FACS lysing solution (Becton–Dickinson Immunocytometry Systems, San Jose, CA) containing 1.5% formaldehyde as a fixative. For proper lysis of the RBC, the blood cells are added directly to the lysis solution and the sample is mixed well in the solution. This treatment ruptures red blood cells, releasing the malaria parasites which are subsequently fixed by the formaldehyde. The white blood cells (WBC) remain intact and are fixed as well. The

samples are lysed and fixed for 30 min at room temperature and are stored in the lysing solution at 4°C. We found that samples can be stored up to a year in this way.

F. Fixation of Infected Blood Cells from Drug Susceptibility Microtests

Microtests are performed in 96-well microtiter plates. To fix the cells in the wells, culture medium is carefully removed from the cells using a micropipette leaving the blood cells at the bottom of the wells. The cells are fixed immediately by adding 200 μl 0.25% glutaraldehyde in PBS. The plates containing the fixed samples can be sealed and stored at 4°C until analysis. In this way material can be kept in fixing solution or in PBS for over 6 months at 4°C without significant deterioration.

G. Staining of Infected Blood Cells and Free Parasites with Hoechst 33258 after Fixation

Samples of fixed infected blood cells or free parasites are stained for 1 hr at 37°C in the dark in 1–2 μM Hoechst 33258 in PBS. In case of the samples containing fixed RBC, part of the sample is diluted with PBS to a volume of 1 ml (approximately 10^6–10^8 RBC/ml). To this suspension 2–4 μl of a 500 μM stock solution of Hoechst 33258 is added. In case of samples containing free parasites, 200–500 μl of the sample is centrifuged for 1 min in an eppendorf centrifuge (15,000*g*) and the supernatant is carefully removed to prevent loss of cells. To the pellet 0.2 to 0.5 ml of a staining solution containing 1 μM Hoechst 33258 in PBS is added, and the suspension is mixed. In the final cell suspension at least 10^5 WBCs/ml should be present. The cells remain in staining solution until analysis, which is usually performed within 0–3 hr after staining.

The same procedure can also be applied to samples from drug susceptibility tests. Fixation solution in the wells is carefully removed using a micropipette and the cells are resuspended in 200 μl staining solution which contains 2 μM Hoechst 33258 in PBS. Cells are stained in the plates at 37°C for 1 hr in the dark.

H. Staining of Free Parasites with Hoechst 33258 in Combination with Propidium Iodide

When low numbers of infected cells are present in blood samples (<0.1%), red blood cells are lysed before staining and analysis by flow cytometry. We have found that staining of the parasites with Hoechst 33258 in combination with propidium iodide improves the capability to distinguish parasites from background fluorescence (see Section III, Results and Discussion) (P. H. van Vianen, unpublished results).

Fixed samples of free parasites (approximately 10^5 WBCs; see above) are centrifuged 1 min (15,000*g*) in an eppendorf centrifuge. The supernatant is removed and replaced with 0.3 ml staining solution containing 2 μM Hoechst 33258 in PBS. After the samples are stained for 1 hr at 37°C in the dark, 0.3 ml propidium iodide solution in PBS is added to a final concentration of 1 μg/ml. The cells remain in this solution at room temperature for 30 min until analysis. Analysis will be performed within 0–3 hr after staining.

V. Critical Aspects of the Preparation and Staining Procedures

In general, for the study of malaria infection, some of the most critical aspects are the preparation and culture of blood stages of the parasites. These procedures have been described in detail elsewhere and do not fall within the scope of this chapter.

Because cell collection and preparation procedures will also occur under primitive conditions in field research in developing countries, these methods must be simple and straightforward. Other prerequisites are that sample handling can be minimized or automated, especially with large numbers of samples, and that samples can be stored for long periods, which is convenient in epidemiological studies. Both cell preparation and staining procedures described here are simple and easy to perform. Washing steps before or after fixation are not essential for reproducible results. The cells can be stored for long periods either in PBS or in fixative.

Fixation of infected RBC with glutaraldehyde (GA) is fast and very easy. However, some problems can occur. Especially when fixing cells in 96-well microtiter plates after removing the culture medium, care must be taken to add the fixative before the cells deteriorate. In all cases the cells must be mixed vigorously with the fixative by shaking, whirl mixing, or using the pipette. Because GA fixation is quick, it can be replaced by PBS after 10–15 min and cells can be stored in PBS. When samples are stored in GA, storage should be at 4°C. When stored in GA for long periods at higher temperatures (ambient temperatures in the tropics), GA can cause an increase in background fluorescence of uninfected RBC, causing overlap with infected RBC.

For optimal lysis of RBC in the preparation of free parasites, the RBC should ideally be suspended directly in the lysis solution after being collected. Preparing and handling of the free parasites after lysis of RBC must be carefully performed. Loss of cells must be prevented during the steps in which the lysing solution is removed and replaced with the staining solution. Since these methods are used to prepare cells for detection and quantitation of low number of parasites, small losses of free parasites or WBC could significantly influence the reliability and reproducibility of the results.

The length of the staining period of fixed cells is not very critical: during analysis which can take several hours, cells are normally kept in the staining solution at room temperature. This appears not to affect the fluorescence intensity of the cells.

VI. Standards

Young blood stages (ring forms and trophozoites) are non-DNA synthesizing haploid organisms, which show a narrow symmetrical distribution of fluorescence values after being stained with Hoechst dyes. With flow cytometric analysis using fixed laser power and fixed amplifier settings, haploid parasites fall within a small region in the fluorescence histogram. In most experiments using fixed RBC, samples containing these haploid stages are used as a standard and for determining the initial settings of the flow cytometer (see also Section VI, Instruments).

Similar to what is described for the analysis of parasites in intact RBC, samples containing free haploid parasites can be used as standard and to determine the initial setting of the flow cytometer (see Section VI, Instruments section).

VII. Instruments

A. Analysis of Samples Containing Hoechst-Stained Infected Erythrocytes

To determine the percentage of infected RBC and DNA synthesis by parasites, samples have been analyzed with a FACS analyzer and with a FACStar (Becton-Dickinson, San Jose, CA). The FACS analyzer is equipped with a mercury arc lamp. Standard filter sets for UV excitation are used: a BP 360 and SP 375 for excitation and a SP 375 as dichroic mirror, and the blue Hoechst fluorescence is selected using a BP 490 and two LP 400 filters. Because the FACStar has a better sensitivity and higher discriminative properties for the light scatter this instrument is preferred and used for most studies reported here.

The FACStar is equipped with a Coherent Innova 90 laser tuned to UV excitation (351 nm, 50 mW). The blue Hoechst fluorescence is selected with a BP 485/22 optical filter. By setting an electronic threshold in the forward angle light scatter (FSC), debris is eliminated from analysis. Tuning and calibration of the FACStar is done using calibration beads containing defined amounts of fluorescent dye (Hoechst). A Hoechst-stained sample containing infected RBC is then used for the initial settings of the machine. These settings are monitored on a two-dimensional dot plot of Hoechst fluorescence and FSC, similar to what is shown in Fig. 4. Since uninfected RBC and infected RBC with single-

haploid parasites, as well as schizonts containing more than 30 nuclei, need to be presented in the same histogram, the fluorescence gain setting is set in a logarithmic scale. The lower threshold in the FSC is set so that free merozoites, which are much smaller than the RBC, are still included.

The fluorescence intensity and FSC of 10,000–50,000 cells per sample are measured, collected in list mode, and stored using the standard BD Consort 30 software. Data analysis can be performed using the Consort 30 software, but for analysis of malaria parasite development in culture, specialized software is developed (see also Section VIII, Results and Discussion).

B. Analysis of Samples Containing Free Parasites Stained with Hoechst 33258 in Combination with Propidium Iodide

To detect and count free parasites, samples are analyzed using a FACStar equipped with a Coherent Innova 90 laser tuned to UV excitation (351 nm, 50 mW). A dichroic mirror (DM560), a BP 485/22 for the blue Hoechst fluorescence, and a LP 620 for the red propidium iodide are selected as emission filters. Calibration of the FACStar is done using calibration beads containing defined amounts of fluorescent dye (Hoechst). A lysed blood sample containing sufficient numbers of parasites is used for the initial settings of the machine. Because both parasites and WBC have to be included in the same fluorescence histograms, the fluorescence gain settings are set in the logarithmic scale. To eliminate small weakly fluorescing particles which are not of interest, an electronic threshold is set in the red fluorescence signal, just below the fluorescence signal of parasites from the control sample. All samples are analyzed using these fixed settings. Of each sample, 5000 events are analyzed and the data are stored using the standard Becton–Dickinson Consort 30 software. For data analysis, parasite and WBC populations are identified and selected by setting a gate in a two-dimensional dot plot of FSC and blue fluorescence. This gate is set using the data from the control sample.

C. Analysis of Samples from Microtests Containing Hoechst-Stained Parasites

The analysis of samples from microtests is as described for the analysis of Hoechst-stained infected RBC by the FACStar as described above. However, the samples are not fed into the flow cytometer by hand, but sampling from the wells is performed automatically using an AutoMATE (Becton–Dickinson). This enables the fully automatic analysis and storage of samples from a complete 96-well microtiter plate. The acquisition is performed using the AutoMATE Control Program (ACP). With the ACP, samples of interest can be selected. Furthermore information for each sample can be added and stored together with the flow cytometric data. This can be used for identification purposes and for data processing by the program described below (Reinders *et al.*, 1994).

The program selects and identifies the events of interest, such as uninfected

RBC, infected RBC, and free parasites by their FSC and fluorescence characteristics. This is used to determine the percentage of infected RBC whereas parasite development is calculated from the fluorescence distribution of the infected RBC which can be expressed as the increase in the mean number of nuclei per parasite (growth) and as the total number of nuclei synthesized, respectively. Results from several samples from the same culture series are combined in graphs and tables (see Fig. 5). Alternatively, results from single samples can also be displayed.

VIII. Results and Discussion

A. Blood Stages: Development and DNA Synthesis

The nuclei of parasites stained with Hoechst dyes show a strong specific fluorescence. Therefore, infected RBC are clearly separated from uninfected cells on the basis of Hoechst-DNA fluorescence intensity (Fig. 2). The assess-

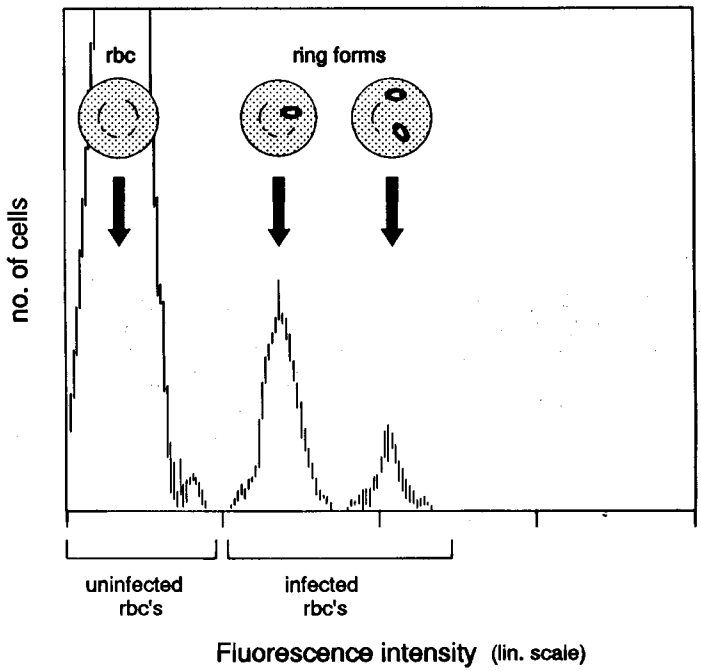


Fig. 2 Schematic representation of a frequency distribution showing the fluorescence distribution of a blood sample containing uninfected RBC and malaria-infected RBC containing haploid non-DNA synthesizing parasites, the ring forms. The RBC were fixed with glutaraldehyde before being stained with Hoechst 33258. Two peaks represent the infected cells: the first peak consists of RBC with one haploid ring form and the second peak consists of RBC infected with two ring forms.

ment of the percentage of infected cells by flow cytometry based on this difference in fluorescence intensity corresponds closely to the assessments by microscopic examination of Giemsa-stained slides (Janse *et al.*, 1987).

Frequency distributions of the fluorescence values of young ring forms and merozoites show narrow, symmetrical Gaussian distributions (see Fig. 2). These stages of the parasite are haploid and non-DNA synthesizing. In the fluorescence histograms of these stages often a small second peak is observed of cells with a double-fluorescence intensity. This peak represents infected cells containing two ring forms (double-infected RBC) or is caused by the simultaneous measurement of two infected cells or two free parasites.

During development of the ring forms into the old trophozoites, parasites increase in size but do not synthesize DNA. Old trophozoites show about 10% higher fluorescence intensity than ring forms, which is due to an increase of a non-specific background fluorescence of the cytoplasm of the parasite (Janse *et al.*, 1987). In the schizont stage of development a rapid increase in DNA content and number of nuclei occurs as the result of three to five mitotic divisions, resulting in the production of 8–32 merozoites per parasite. The increase in the number of nuclei is proportional to the increase of the fluorescence intensity of Hoechst-stained schizonts (Fig. 3). Therefore, the frequency distributions of the fluorescence intensities of a population of (dividing) stages at different time points are representative of the development and degree of DNA synthesis of the parasites (Janse *et al.*, 1987). These frequency distributions can be used to determine the inhibition of parasite development and DNA synthesis by antimalarial drugs (see below).

B. Determination of Drug Susceptibility of Parasites

Determination of antimalarial activity of drugs *in vitro* which inhibit development of the blood stages can be performed routinely using flow cytometry. Here we describe results from experiments using two different species, *P. falciparum* and *P. berghei*.

1. *P. berghei*

Young ring forms are cultured for 24 hr under standard culture conditions in RPMI 1640 medium to which different concentrations of the drugs are added. Blood-stage development of *P. berghei* from haploid ring forms to the mature schizonts containing 8–24 nuclei takes 22–24 hr. RBC containing the mature schizonts do not burst spontaneously in culture, but remain intact and viable for several hours. Samples for flow cytometry are taken from these cultures, before the culture is started and after 24 hr. Samples at the start of the cultures contain ring forms which show a narrow symmetrical frequency distribution of their fluorescence intensities (Fig. 2). The mean fluorescence intensity of ring forms/young trophozoites represents the haploid DNA content and can be used

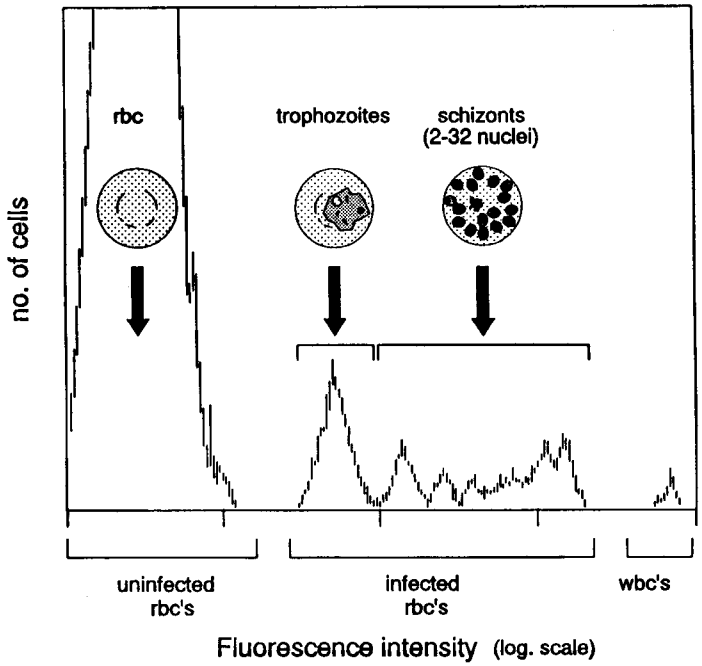


Fig. 3 Schematic representation of a frequency distribution showing the fluorescence intensity of a sample containing uninfected RBC and infected RBC. Parasites range from haploid non-DNA synthesizing trophozoites to mature schizonts containing 16–32 merozoites with immature schizonts in between. The RBC were fixed with glutaraldehyde before being stained with Hoechst 33258. The fluorescence gain setting is set in a logarithmic scale. The small peak with the highest fluorescence intensity represents the white blood cells (WBC). From van Vianen *et al.* (1990b), with permission.

as an internal standard to calculate the number of nuclei in the schizonts. In the 24-hr sample from cultures without antimalarial drugs parasites show fluorescence values between 1 and 24 times the haploid amount. This comprises mature schizonts with 8–24 nuclei, immature schizonts in the process of DNA synthesis, and some degenerated parasites and free merozoites which are liberated from the RBC during handling of the samples. Figure 3 shows a schematic representation of a histogram of the fluorescence distribution of a sample containing trophozoites and schizonts. Figure 4 shows a two-parameter dot plot representation of flow cytometric data showing fluorescence intensity and FSC of a sample containing trophozoites, schizonts, and free merozoites.

Based on the mean fluorescence intensity of the cells, the software developed for this purpose calculates the percentage of infected cells and the total number of nuclei and the average number of nuclei per parasite present. Additionally the parasite growth and DNA synthesis of the whole series are calculated and

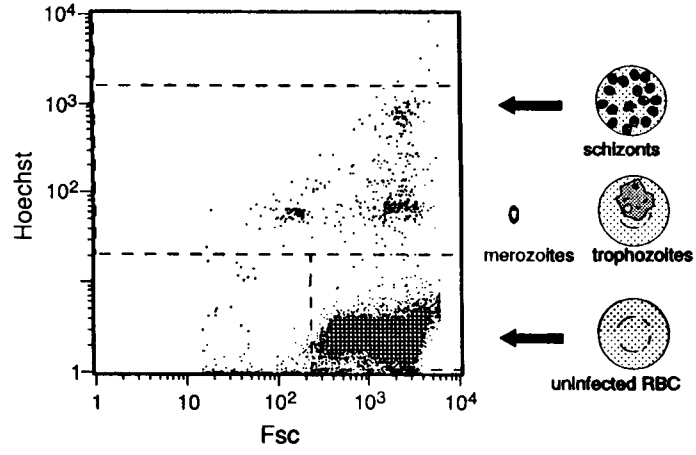


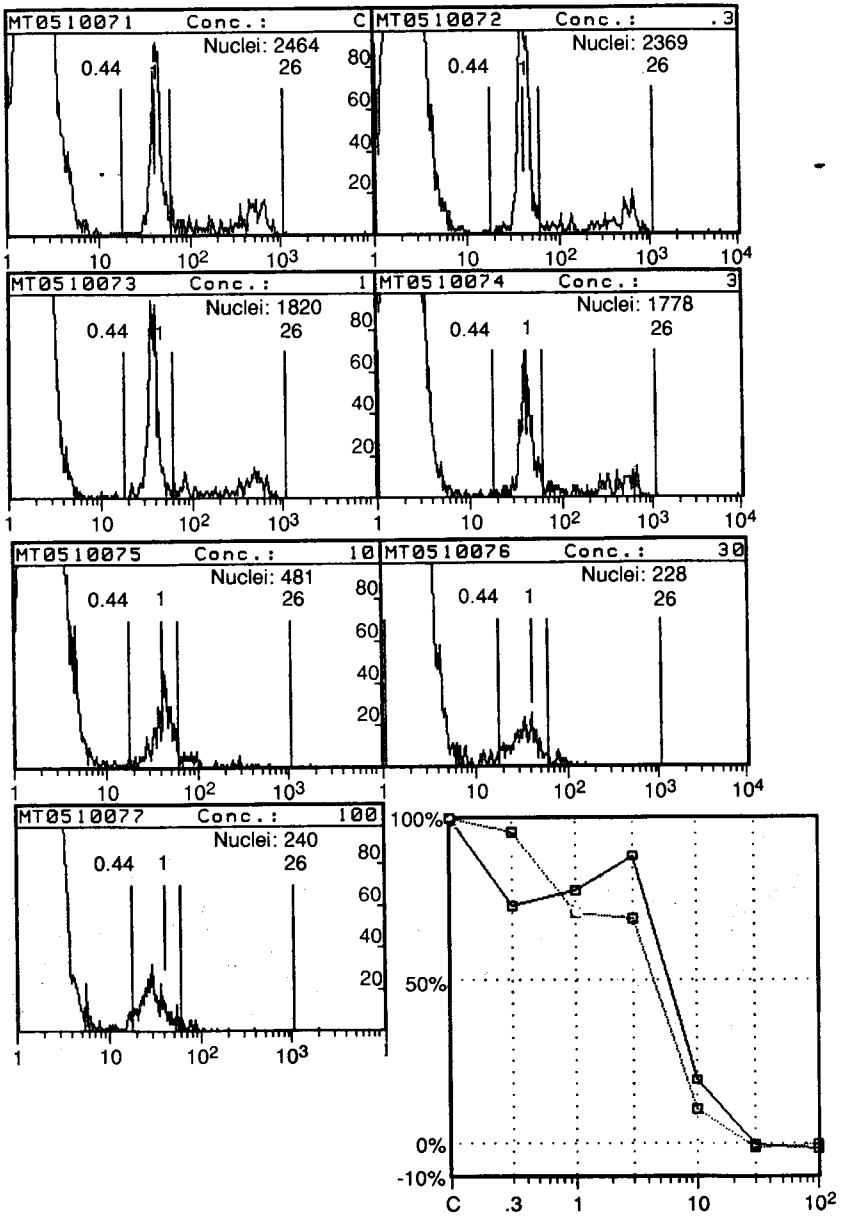
Fig. 4 Two-parameter dot plot representation of fluorescence intensity and FSC from a sample containing uninfected RBC, free merozoites and infected RBC containing trophozoites, and immature and mature schizonts. The cells were fixed with glutaraldehyde before being stained with Hoechst 33258.

presented in a graph and table. Parasite growth is defined as the average number of nuclei/parasite in a sample divided by the maximum average number of nuclei/parasite in the cultures. DNA synthesis is defined as the total number of nuclei in a sample divided by the maximum total number of nuclei in the cultures. Figure 5 shows an example of the calculation of parasite growth and DNA synthesis in samples obtained from cultures containing different concentration of an antimalarial drug. Figure 6 gives an example of the flow cytometric comparison of inhibition of parasite growth/DNA synthesis by three related antimalarial drugs. The results obtained by flow cytometry are comparable with results obtained by microscopic examination of parasites in Giemsa-stained slides (Janse *et al.*, 1987; van Vianen *et al.*, 1990a) or by measurement of the incorporation of radioactive precursors into RNA/DNA (C. J. Janse, unpublished results).

2. *P. falciparum*

Different *in vitro* tests have been described for determination of drug susceptibility of *P. falciparum* parasites. Parasites, often ring forms or young trophozo-

Fig. 5 Results from the computer program which calculates parasite inhibition by an antimalarial drug in a series of cultures. Each histogram represents the fluorescence distribution of parasites cultured without drug (control) or in the presence of different concentrations of the drug ranging from 0.3–100 ng/ml. In this experiment *P. berghei* ring forms were cultured for 22 hr in the presence of sodium artesunate after which samples of the cultures were fixed with glutaraldehyde and stained with Hoechst 33258. From the histograms it is clear that ring forms still develop into (mature)



schizonts at low concentrations up to 3 ng/ml. No schizonts can be detected at 30–100 ng/ml. The first peak in the histograms contains ring forms/free merozoites and trophozoites and the mean fluorescence intensity of these cells represents the haploid amount of DNA (one nucleus). This value is used to calculate the total number of nuclei in the different samples which is shown in the upper right corner of the histograms. The graph shows the inhibition of growth (. . .) and DNA synthesis (———). (See also Section VII.B.)

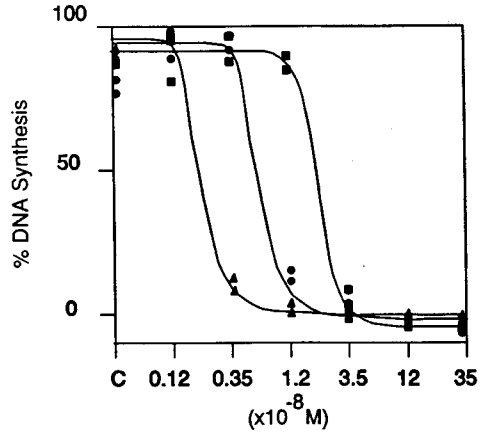


Fig. 6 An example of comparing the antimalarial activity of three related drugs, artemisinin (■), dihydroartemisinin (▲), and sodium artesunate (●) as measured using flow cytometry. In this experiment ring forms of *P. berghei* were cultured for 22 hr in the presence of different concentrations of the three drugs. At the start of the cultures and after 22 hr, samples were taken from the cultures, fixed with glutaraldehyde, and stained with Hoechst 33258. The total number of parasite nuclei in the samples is calculated as described in the text of Section VII,B. DNA synthesis is defined as the increase in number of parasite nuclei during the culture period.

ites, are cultured for prolonged periods (48–96 hr) in the presence of different concentrations of drugs. The length of the culture periods depends on the process studied (e.g., reinvasion or schizont development) and on the method used to determine parasite development. In Giemsa-stained slides differences in the number of infected cells compared to the control culture are counted or the number of DNA synthesizing parasites (schizonts) is determined as parameters for parasite development. Alternatively the incorporation of radioactive precursors into the DNA of parasites can be measured. The development of ring forms into mature schizonts takes 48 hr, after which schizonts burst spontaneously and the free merozoites enter new RBC. In cultures without drugs this will result in the increase of the number of infected cells. Figure 7 gives an example of determination of the chloroquine susceptibility of *P. falciparum* in the "extended 72-hr test" by flow cytometry.

A standardized test to determine drug susceptibility is the WHO microtest. Here parasites are cultured for 26–30 hr in 96-well microtiter plates, which are predosed with different concentrations of antimalarial drugs. During this culture period ring forms or young trophozoites develop into (immature) schizonts. The percentage of schizonts after culture is used for the assessment of susceptibility. Since flow cytometry can rapidly and reproducibly determine the increase of the number of nuclei a method has been developed for automated flow cytometric analysis of drug susceptibility of *P. falciparum* in microtests. For this purpose the AutoMATE is used for automatic sampling from the plates for flow cyto-

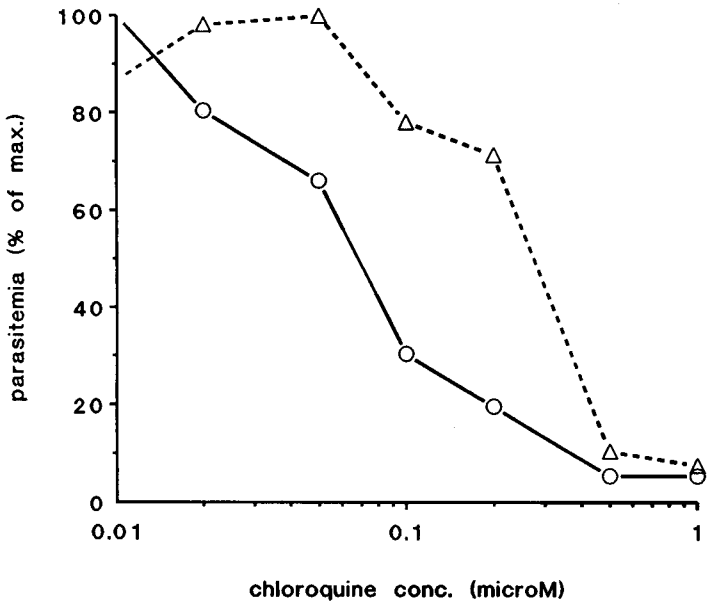


Fig. 7 Comparison of the chloroquine susceptibility of *P. falciparum* isolates (○ = clone T9/94; △ = isolate TM 152) in the "extended 72-hr test" using flow cytometry. In this test parasites are cultured for 72 hr in the presence of different concentrations of chloroquine and inhibition of development is determined by measurement of the increase/decrease of the number of infected cells during the culture period. Here the number of infected cells is determined by flow cytometry after fixed (infected) cells are stained with Hoechst 33258. In 63 tests it was found that the results obtained by flow cytometry closely corresponded with results obtained by microscopic examination of Giemsa-stained slides (van Vianen *et al.*, 1990b). From van Vianen *et al.* (1990b), with permission.

metric measurements. Automatic sampling and flow cytometric analysis of a complete 96-well plate takes 2 hr. Data analysis is performed with the described software which calculates the percentage of infected cells, the total number of nuclei per sample, and the average number of nuclei per parasite in each sample. Parasite growth and DNA synthesis for a complete culture series is calculated as described above. Figure 8 shows an example of the determination of chloroquine susceptibility of *P. falciparum* in microtests by flow cytometry compared to results by microscopic examination. We have shown in several experiments that automatic reading of a microtest by flow cytometry gives results comparable with those of microscopic examination of Giemsa-stained slides (van Vianen *et al.*, 1990b; P. H. van Vianen, unpublished results).

C. Detection and Counting of Low Numbers of Parasites

Since infected cells show a higher fluorescence intensity than noninfected cells, the percentage of infected cells can be established by flow cytometry.

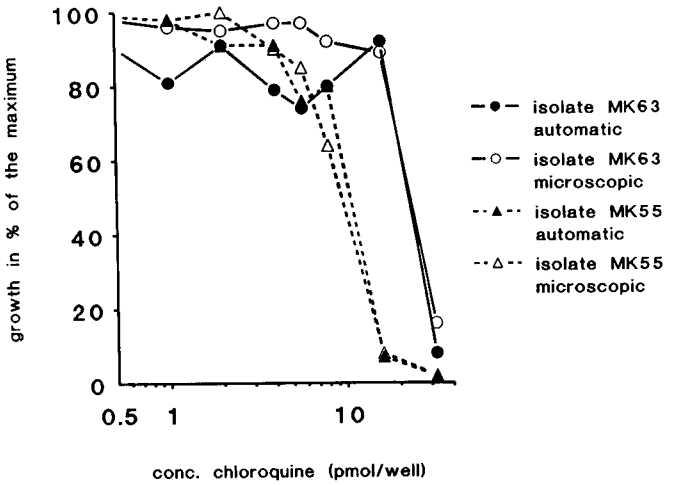


Fig. 8 Comparison of chloroquine susceptibility of two *P. falciparum* isolates in standard drug susceptibility microtests. Inhibition of development was determined either by flow cytometric analysis or by microscopic examination of Giemsa-stained thick smears. Parasites were incubated in 96-well microtiter plates under standard culture conditions for a period of 30 hr. These plates were predosed with different concentrations of chloroquine. After 30 hr samples were taken to prepare slides for microscopic examination. The rest of the culture material was fixed in the plates with glutaraldehyde and stained with Hoechst 33258. Flow cytometric reading of the plates was performed using the AUTOmate and parasite growth and DNA synthesis was calculated as described in Section VII,B. Calculation of parasite growth by microscopy is done by dividing the number of schizonts counted by the total number of parasites counted. From van Vianen *et al.* (1990b), with permission.

When this percentage is higher than 0.1%, reproducible counts are obtained in samples where the blood cells are fixed before staining and measurement. However, less reproducible results are obtained with fixed RBC when the percentage is lower than 0.1%, due to the presence of low numbers of RBC or reticulocytes in the blood samples, which show aspecific background fluorescence. Their fluorescence intensity is in the same range as that of infected cells and they may interfere with the measurement. Therefore, to detect low numbers of parasites in blood samples, the RBC are first lysed before fixation of the parasites. Figure 9 shows the fluorescence intensity and the FSC of a blood sample, which is lysed before fixation and staining with Hoechst 33258. Three populations can be distinguished: WBCs, platelets, and parasites. From dilution experiments, in which infected blood was diluted with uninfected blood, we have found that *P. falciparum* parasites were reproducibly detected at a percentage of about 0.005% (Fig. 10). Using this method, the detection of *P. berghei* is somewhat less sensitive due to interference by residual bodies of the nucleus in a low percentage of rodent RBC. These residual bodies do not lyse and can

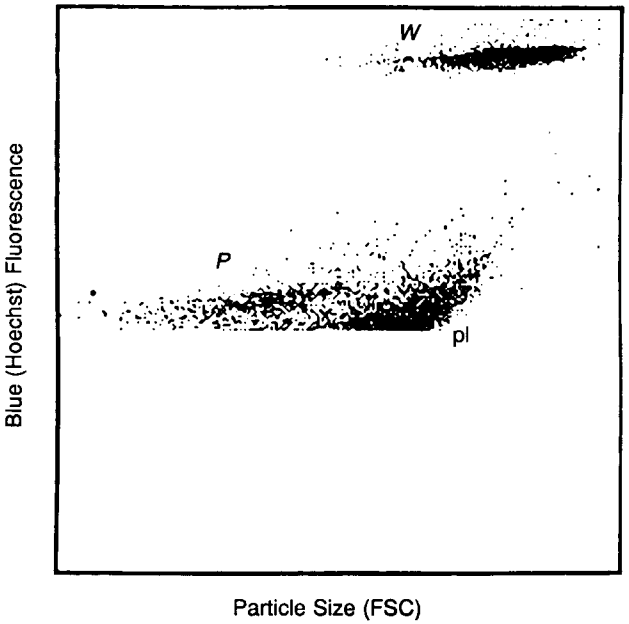


Fig. 9 Two-parameter dot plot representation of fluorescence intensity and FSC from a blood sample after lysis of the RBC in FACS lysing solution, fixation with formaldehyde, and staining with Hoechst 33258. The blood sample is from a patient infected with *P. falciparum* with a percentage of infected RBC of about 0.01%. Three populations of cells can be distinguished: white blood cells (W), platelets (pl), and parasites (P). From van Vianen *et al.* (1993), with permission.

show fluorescence intensities comparable to that of parasites (see the relative high number of "parasites" in noninfected blood samples in Fig. 10).

The reproducible detection of lower numbers of parasites is hampered by the fact that the fluorescence intensity and FSC of a low percentage of platelets fall in the same range as those of the parasites. We found that the combination of Hoechst 33258 and propidium iodide staining of the lysed blood samples allows a better separation of platelets and parasites. When the samples are stained with propidium iodide alone parasites cannot be separated from platelets on the basis of the red fluorescence. In combination with Hoechst 33258 staining, however, the red fluorescence of the parasites significantly increases while the fluorescence intensity of the platelets remains the same, allowing a better separation of those two populations. It is suggested that energy transfer from DNA-bound Hoechst 33258 to propidium iodide takes place, by which the red fluorescence of the parasites is more enhanced than that of platelets which do not contain DNA. This staining method has been used in clinical and epidemiological studies to detect low numbers of parasites in blood samples from patients. We

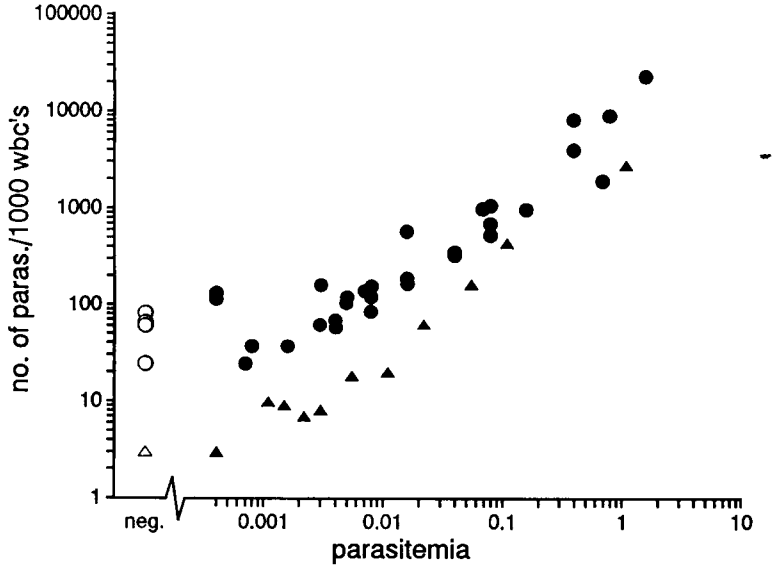


Fig. 10 Relationship between the number of parasites counted by flow cytometry and the expected percentage of infected cells (parasitemia) in blood samples. In these experiments infected blood was serially diluted with noninfected blood after which RBC were lysed and the parasites fixed and stained with Hoechst as described in Fig. 9. Parasite numbers counted by flow cytometry (in parasites per 1000 WBCs) are compared with the expected parasitemia calculated from the starting parasitemia and the dilution factor. (*P. berghei*, O; *P. falciparum*, △; open symbols are from blood samples containing no parasites). From van Vianen *et al.* (1993), with permission.

found that as few as 20 parasites per μl of blood (equivalent to 0.0004% infected RBC) could be detected (P. H. van Vianen, unpublished results).

IX. Comparison of Methods

A. Drug Susceptibility of Parasites

The use of an instrument such as a flow cytometer for the analysis of samples collected under primitive field conditions in developing countries seems contradictory to the necessity to keep field procedures uncomplicated. However, the collection of samples in the field is very simple. For drug susceptibility tests only the addition of fixative to the culture wells is essential. In fact the procedure is more easy than making smears of the culture material. Compared to other techniques, flow cytometric analysis of microtests provides more information. With microscopic examination of thick smears only the percentage of schizonts is counted and with the use of radioactive precursors only the total amount of

precursor in the nucleic acids per sample is used to establish parasite development. Flow cytometry enables the combination of a number of different parameters. Information on the percentage of infected cells, the schizogonic development of individual parasites, extra- versus intraerythrocytic parasites, and invasion of new RBC can be generated in one analysis. An additional improvement is the automation of the analysis and processing of the data. All data are processed in a standardized way, maintaining objectivity, and the data remain available for reexamination. Data from large numbers of tests can be combined.

B. Detection and Counting of Parasites

Routine diagnosis of malaria is generally based on examination of Giemsa-stained blood smears for blood-stage parasites. Since this disease requires a rapid treatment, ideally within several hours after attending the hospital, a rapid sensitive and simple diagnostic method is required. This is provided by the Giemsa-stained smear examination. Since flow cytometric analysis as described in this chapter requires a FACStar flow cytometer, the method is less suitable for direct diagnosis of malaria, especially in hospitals in developing countries. However, flow cytometric analysis has proven to be a sensitive, rapid, and reproducible method when large numbers of samples have to be screened for malaria parasites in epidemiological studies or clinical studies. It allows for the reproducible detection of less than 20 parasites per microliter of human blood (0,0004% infected RBC). This is comparable to the sensitivity obtained under optimal laboratory conditions by the use of radioactively labeled DNA probes specific for malaria parasites. Nonradioactive methods are less sensitive. The sensitivity of microscopic examination is reported to be better. In optimal circumstances, 1 parasite per μl blood (0.00002% parasitemia) can be detected, although 10–20 parasites per μl is probably more realistic. When both the probe and the microscopic method were tested under field conditions their sensitivities appeared to be much lower. In these circumstances, both radioactive DNA probes and microscopy were reported to approach their limits of reproducible detection at parasitemias of 0.016% in the field (Barker *et al.*, 1989). Flow cytometric analysis is not affected by the circumstances in which the samples are collected and handled, and the same sensitivities have been found in the laboratory and with field samples. Furthermore flow cytometry allows for quantitation of the number of parasites. This is not easily performed with other methods. In conclusion, flow cytometry seems to be the best technique available for the detection and quantitation of parasites in large numbers of blood samples. Especially in view of the recent interest in vaccine development and the use of newly developed drugs this method is suitable for the follow-up of patients after treatment or the follow-up of vaccine trials and for large-scale epidemiological research in malaria.

Acknowledgments

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