Introduction

Between 300 and 500 million new cases of malaria are diagnosed worldwide every year, 90% of them in Africa. However, a growing number of cases are diagnosed in Europe and America, mainly as a result of the increase in tourist and cultural travel to endemic areas. In European countries an average of 60% of the cases of malaria are misdiagnosed with routine techniques, a situation that leads to a delay of 8–10 days in adequate diagnosis of the disease (Kain et al., 1998). This is largely because physicians in non-endemic areas do not usually consider malaria in the differential diagnosis of fever, and rarely request a complete haematological study with specific techniques for the diagnosis of malaria.

An increasing interest therefore exists in using the results of routine haematological tests as a tool for the presumptive diagnosis of malarial infection (Hänscheid et al., 1998). In this setting, several authors have recently reported that the depolarization test using the Cell-Dyn 4000 analyser as an integral part of the leukocyte differential count is a useful diagnostic tool (Ben-Ezra et al., 1999; Jones et al., 2001).

The analytical principles of the Cell-Dyn 4000 analyser are the same as those upon which flow cytometry is based (Grimaldi & Scopacasa, 2001). The leukocyte differential is obtained by measuring the amount of laser light dispersed by a cell at different angles (0°, 7°, 90° polarized and 90° depolarized). Dispersed light depolarized at 90° enables eosinophil identification and counting, since these are the only cells capable of depolarizing light, due to the presence of their cytoplasmic granules. However, malaria samples also contain other abnormal cell populations with depolarization capacity. This is due to leukocytes with a phagocytic capacity engulfing the haemozoin pigment that is produced by the parasite as it degrades the haem group of haemoglobin. Haemozoin depolarizes light, and therefore transfers this depolarization capacity to the leukocytes that have incorporated the pigment into their cytoplasm (Mendelow et al., 2000; Krämer et al., 1999). The purpose of this study was to evaluate the sensitivity of light depolarization analysis conducted with the Cell-Dyn 4000 analyser for the detection of patients infected with *Plasmodium*.

Methods

Tests were performed on 411 peripheral blood samples collected in tubes containing EDTA from subjects with suspected malarial infection using a Cell-Dyn 4000 analyser (software revision R8-2). The tests were performed according to the conventional analytical configuration no more than 5 h after blood drawing. Following analysis, the leukocyte differential ‘NEU-EOS’ (90° light dispersion versus 90° depolarized) and ‘EOS I’ (90° depolarized versus 0°) graphs were studied.

Another parameter automatically provided by the analyser and used in this study, apart from red blood cell (RBC) and platelet counts, which
are usually low in malarial infections, was the immature reticulocyte fraction (IRF). The system incorporates fluorescent staining of reticulocyte RNA, which gives information about the IRF, representing the proportion of immature reticulocytes. Depending on their RNA content, they yield a greater or lesser intensity of fluorescence staining (FL1 fluorescence channel). Consequently the analyser detects RBCs containing large amounts of RNA, such as those infected with Plasmodium, as these are represented as an extra signal (‘peak’) on the graph of the FL1 fluorescence channel (corresponding to RNA staining).

Malarial infection was diagnosed by Field staining of both thick and thin blood smears, followed by parasite visualization under the microscope. The microscope-negative/positive samples were confirmed by PCR.

**Results**

A total of 39 samples proved positive for malarial infection by microscopy, representing 9-5 % of all samples included in the study; of these positive samples, 35 corresponded to Plasmodium falciparum, three to Plasmodium ovale and one to coinfection with P. falciparum and Plasmodium vivax (Table 1). The parasitaemia range of these positive samples was < 0-1 % to 4-6 %, with a mean of 1-0 %. Gametocytes were seen in five cases. Virtually all the patients with malaria were black subjects from Equatorial Guinea. The mean age was 42 years, but there were two children aged 2 and 4 years.

The mean IRF value was 0-35, and all positive samples had IRF values less than 0-5, except for five samples in which the IRF value exceeded this limit. These five samples showed the highest blood parasite levels (2-2–4-6 %) and were the only samples that showed an additional peak that appeared to suggest the existence of malarial infection. This could be due to the presence of younger and fewer circulating parasites, which produced less haemozoin and were below the analyser detection limits.

The presence of one or more purple depolarizing events (monocytes) on the NEU-EOS graph, was taken to be a possible indication of the existence of malarial infection. Twenty-eight of the 39 positive samples could be detected in this way, which indicates a sensitivity of 72 %. The specificity reached 98 %, whereas the positive and negative predictive values were 78 % and 97 %, respectively.

When samples were studied comparing the number of infected RBCs we observed a reduction in sensitivity down to 66-66 % with parasitaemia in the range 0-1–1 %, and to 50 % with less than 0-1 % RBCs infected.

**Discussion**

In the early 1990s, multiple reports appeared describing the existence of atypical patterns in the leukocyte population graphs of patients with malaria, primarily associated with 90° dispersion of depolarized light. During the intraerythrocytic stage, the malaria parasite digests the haemoglobin and converts the haem group into haemozoin. Unlike haemoglobin, haemozoin depolarizes light; as a result, after haemozoin is released from the RBCs it is phagocytosed by monocytes and neutrophils, which allows these two cell populations to depolarize light, a situation not found under normal conditions.

Based on this phenomenon, the sensitivity and specificity of the Cell-Dyn 4000 system for detecting these depolarizing abnormal cell populations were 72 % and 98 %, respectively. These figures are similar to those reported by other authors using the Cell-Dyn 3500 analyser (72 % and 96 %, respectively) (Mendelow et al., 2000). However, in that study a lower sensitivity was noted for detecting malaria in Caucasians than in black populations. We were unable to confirm this difference, since all our infected patients were black. A remarkable reduction in sensitivity was noted with low parasitaemia levels, probably due to the presence of younger and fewer circulating parasites, which produced less haemozoin and were below the analyser detection limits.

Virtually all the positive samples had IRF values within the normal range, except for samples with high blood parasite levels. In addition to this high IRF value, these samples showed an additional peak that appeared to suggest the existence of reticulocytosis. However, this ‘pseudo-reticulocytosis’ is due to the affinity of the CD4K530 fluorochrome for DNA (Scott et al., 2001). The perceived fluorescence signal corresponds to the DNA of the parasite located inside the erythrocytes. This is similar to what occurs with the DNA found inside Howell-Jolly bodies.

An interesting observation was that all five samples corresponding to P. falciparum in the form of gametes showed a set of green events (eosinophils) on the NEU-EOS graph, leading to a green cloud more or less in the same location as the purple events. However, the EOS I graph showed a vertical cloud of green or black events, drawing a totally atypical pattern not seen in any other samples in which the parasite was in non-gamete form. This could be due to the unique properties of the gametes in relation to light dispersion and to their haemozoin-rich internal contents (Mendelow et al., 2000).

Fig. 1 illustrates the above comments, corresponding to the follow-up of a 36-year-old patient from Equatorial Guinea with malaria and the course of the disease with treatment. Upon arrival to hospital (Fig. 1a), the patient had a

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**Table 1. Results of blood samples examined by microscopy and laser light depolarization analysis**

<table>
<thead>
<tr>
<th>Parasite and level of parasitaemia</th>
<th>No. of positive samples</th>
<th>Microscopic examination</th>
<th>Depolarizing events</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. falciparum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1 % RBCs infected</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>0-1–1 % RBCs infected</td>
<td>12</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>&lt; 0-1 % RBCs infected</td>
<td>14</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Gametocytes</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>P. ovale</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1–1 % RBCs infected</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Mixed infections P. falciparum/P. vivax</td>
<td>1</td>
<td>1</td>
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</tr>
</tbody>
</table>
parasitaemia level of 2.2 %, with an IRF value of 0.64 and a clear ‘pseudo-reticulocytosis’ peak with some purple events on the 90° versus 90° depolarized graph. Six days after the start of treatment (Fig. 1b), the ‘pseudo-reticulocytosis’ peak virtually disappeared, parasitaemia decreased to 1.2 % and the IRF dropped to 0.49. Purple events were no longer seen, probably due to the disappearance of monocytes with haemozoin inside. Such disappearance is complete 2–3 weeks after eradication of the parasite in cases of high parasitaemia (Hänscheid et al., 1998). In our case, since
initial parasitaemia was quite low, the disappearance of these monocytes may have occurred faster. Finally, after another 6 days (Fig. 1c), the most remarkable finding was the presence of a new abnormal population both in the NEU-EOS and EOS I graphs, corresponding to the observation of gametes under the microscope, while the IRF decreased to a normal value of 0.36. This patient could be an example of how the analyser shows the course of malarial infection, with transition from the ring to the gamete form.

As has been seen in this study, the sensitivity of the Cell-Dyn 4000 analyser is not adequate for considering haematological testing as an alternative to the existing methods for diagnosing parasitic diseases. However, we do consider it highly useful to perform a more careful examination of the graphs provided by the analyser when a routine leukocyte count is made. Certain abnormal leukocyte subpopulation patterns could alert us to the unsuspected presence of parasitic infections, as shown by this study in patients infected with Plasmodium.

References


