THE USE OF FLUOROCHROMES FOR THE DETECTION OF MALARIA PARASITES

by

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INTRODUCTION

Fluorochrome stains comprise a group of acid and basic dyes that are excited to fluorescence by near ultra-violet light. The use of fluorochromes as a diagnostic method first came into prominence with Hagemann's method for detecting Mycobacterium tuberculosis. Patton & Metcalf and Metcalf reported on the staining ability of a number of fluorochromes, using both avian and human malaria. Metcalf pointed out the clarity of the malaria parasite, in both thick and thin blood films, when stained with fluorochromes. Berberine sulfate and rivanol gave the maximum fluorescence. Their method, however, stained leukocytes the same colour as parasites.

The purpose of this study was to develop a method for rapid detection of malaria parasites in thick blood films from individuals with low level or subpatent parasitaemia. Such a method might increase the efficiency of screening blood films and replace the cumbersome concentration methods currently available for detecting malaria parasites at low levels. In the design of such a technique, it was highly important that parasites might be distinguished easily from normal blood constituents using a 10X objective. The sensitivity of the fluorescent microscope and the high contrast it develops with a low concentration of fluorochrome seemed to provide a tool capable of fulfilling this objective.

MATERIALS AND METHODS

Seven fluorochromes were initially evaluated to determine which acted as polychromatic stains. Acridine orange, acridine yellow and acriflavine fell into this group. No advantage was seen in one stain over another, except that acridine orange is widely and readily available. For this reason acridine orange was used in the studies reported here.

Thick or thin blood films were prepared using several species of Plasmodium from human and simian sources. Thin blood films were fixed in methyl alcohol for five minutes. Thick blood films were handled in three ways: first, some were dehaemoglobinized and then fixed in methyl alcohol; second, some were fixed in methyl alcohol without first dehaemoglobinizing; and third, some were dehaemoglobinized during staining. Blood films were stained in an acridine orange solution, with concentrations ranging from 0.001% to 1.0% (w/v). The desired concentrations were made by diluting an aqueous 1.0% (w/v) stock solution with Sorensen's phosphate buffer, 0.067 molar. The stock solution should be stored in an amber bottle and allowed to ripen four weeks. The pH was adjusted over a range of five to seven and staining time varied from 1 second to 10 minutes. Blood films were stained, then dipped several times

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into buffer and left to rinse in a separate jar of buffer for 1 to 6 minutes. They were then dipped into distilled water to prevent phosphate crystallization on the surface. Blood films were examined using a Zeiss fluorescent microscope with an Osram HBO 200W mercury arc light source, BG-38 heat filter, BG-12 exciter filter and a Zeiss No. 53 barrier filter with a cut-off at approximately 525 mJ. Following the study of the acridine orange preparations, they were stained with Giemsa and re-examined.

Additional studies were done using combinations of fluorochromes or blood films prepared from whole blood containing common anticoagulants.

RESULTS

From the studies in which blood films were stained at varying pH, concentration and time, the following procedure is selected because it affords the maximum fluorescence with parasite nuclear-cytoplasmic differentiation and the best separation of parasites from normal blood constituents. With care, a thick and thin blood film can be stained on the same slide. The thin film is fixed in absolute methyl alcohol; the thick film is stained without dehaemoglobinization. The stain is a 0.01% (w/v) solution of acridine orange in Sorensen's buffer at pH 5.4. The thick film is first stained for 30 seconds, and then the thin film for 1 second. The slide is then dipped into buffer several times to remove excess surface stain, then placed in a second wash, also of buffer, for 3 minutes and then dipped several times into distilled water.

In the thick blood film stained by this method, parasites fluoresce bright orange-red against a light green background when observed through a 10X objective and 8X eye pieces. Polymorphonuclear leukocytes and platelets fluoresce lime green and lymphocytes have large green nuclei with narrow rims of orange cytoplasm. Through a 40X objective the cytoplasm of parasites fluoresces orange-red and the nucleus green. The morphology is similar to that seen in Giemsa preparations. The parasites and blood components in thin blood films stain in a similar fashion to thick blood films and, in addition, reticulocytes have a red hue and nucleated red blood cells fluoresce yellow. Mature red blood cells are unstained, but the size of the parasitized red blood cell is evident. Schüffner's stippling cannot be seen, and pigment can be visualized only by interchanging the Zeiss No. 53 barrier filter for a filter with a cut-off at approximately 450 mJ.

Fixed thick blood films stain well and have the least background interference. When sodium citrate and sodium tetra-ethylenediamine tetra-acetate (EDTA) are used as anticoagulants the intensity of fluorescence is decreased, but this can be compensated for by increasing the staining time. The addition of heparin and oxalates does not alter the staining intensity. There is no advantage gained by combining two or more fluorochromes. The addition of phenol to prevent formation of mold in the stock solution does not alter the quality of staining. Acridine orange dye from different sources and different lots when used freshly prepared, results in marked variations in the intensity and staining reaction of leukocytes. This variation can be compensated for by allowing the stock solutions to ripen four weeks. Slides stained with acridine orange can be restained satisfactorily with Giemsa without prior destaining.

DISCUSSION

The fluorochrome acridine orange is capable of multicoloured fluorescence. This metachromasia depends upon its binding with nucleic acids (Armsrong, 1956). When stained with acridine orange DNA fluoresces green and RNA orange. This feature permits differential fluorescence of parasites and leukocytes since the RNA of the cytoplasm of the malaria parasite at the prescribed pH and concentration of acridine orange is more intensely stained than that of the leukocytes. It is the intensity of the fluorescence and the differential fluorescence between parasite and leukocyte that permits detection of malaria parasites while rapidly
scanning large areas of blood films using low magnification. One searches for orange-red fluorescing points; when these are detected under low magnification, they are then examined under higher magnification and the morphology confirmed. As in any staining technique, certain artefacts do occur and other micro-organisms or parasites may fluoresce. It is, therefore, recommended that the parasite be marked and the slide stained with Giemsa for confirmation and diagnosis of the species.

In making the preparations for examination, the thick blood film should be slightly thinner than customary. For minimum background fluorescence it is important that the film be dehaemoglobinized during staining rather than beforehand. The pH and concentration of the stain are critical and the outlined procedure must be followed rigidly.

The advantage gained by this method in malaria case detection lies in its ability to locate a malaria parasite rapidly even at very low levels. Such a method could allow more efficient evaluation of the effects of malaria eradication programmes. It could prove useful in detecting partially treated malaria where there is a low level of circulating parasites, in following up cases of malaria to detect recrudescence, particularly in treated cases of drug resistant malaria, and in identifying blood donors as carriers of malaria. Within this framework the fluorochrome technique should prove helpful in the detection of malaria.

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**RESUME**

Le présent document décrit une méthode aux fluorochromes applicable à la détection rapide de faibles quantités de parasites du paludisme dans les étalements sanguins. La méthode consiste à prendre des étalements épais sans déshémoglobinisation préalable ou des étalements minces fixés à l'alcool méthyléque et à les colorer avec la solution d'acridine orange à 0,01 % (P/V) dans une solution tampon de Sorensen à pH 5,4 pendant 30 secondes et 1 seconde, respectivement. On plonge ensuite les lames colorées à plusieurs reprises dans la solution tampon afin d'éliminer l'excès de colorant, puis on les lave une deuxième fois pendant 3 minutes dans la solution. Elles sont ensuite plongées plusieurs fois dans de l'eau distillée.

Dans l'étalement épais coloré selon cette méthode, les parasites du paludisme prennent une fluorescence rouge orange vif sur fond vert clair. Les leucocytes polynucléaires et les plaquettes prennent une fluorescence vert citron et les lymphocytes présentent un grand noyau vert avec une mince bordure de cytoplasme orange. On a utilisé pour l'examen des étalements colorés un microscope Zeiss à fluorescence avec, comme source d'éclairage, un arc de mercure Osram HBO 200 W, ainsi qu'un filtre anticalorique BG-38, un filtre d'excitation BG-12 et un filtre d'arrêt Zeiss 53 avec une limite d'environ 525 mr.

L'avantage de cette méthode est qu'elle permet de déceler facilement les plasmodiums avec un objectif 10X et un oculaire 10X. Pour l'identification des espèces, on peut colorer à nouveau au Giemsa les lames déjà colorées à l'acridine orange.

Nous suggérons d'utiliser cette méthode pour l'examen rapide de lames de sang présentant une faible parasitémie.
REFERENCES


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