Laboratory techniques

Use of dark ground microscopy in haematology

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SUMMARY The novel combination of vital staining, quick drying of thin films, and dark ground microscopy furnishes new information about organelles, granules, and precipitates in blood cells. The preparations are permanent and give optical signals of high contrast which depend on refraction or dichroism of the object. Mitochondria are readily shown and can be counted and sized. Pilot observations suggest that mitochondria in red cells may give information about cell maturity and splenic function; that the size of lymphocyte mitochondria may reflect the state of activation of those cells; and that the sensitivity of unstable haemoglobin detection may be greatly enhanced. The method is unsuitable for the study of granulocyte mitochondria.

Dark ground microscopy has been little used in haematology and rarely on dried material. Its use for the examination of reticulocyte preparations¹ gave such striking effects that we were led to explore other applications in haematology.

Dark ground microscopy differs from the bright-field method in that the refractile properties of the object are exploited rather than suppressed (by "clearing"); contrast is higher; dyed objects show a colour complementary to that seen under brightfield.

In this paper we report the dark ground demonstration of the following: (in unstained preparations) malarial parasites; red cell inclusions; neutrophil and eosinophil granulocytes; iron granules; melanin.

(In vitally stained preparations) mitochondria; Heinz bodies; unstable haemoglobin precipitates; platelet granules; basophil granulocytes; trypanosomes; reticulocytes.

(In fixed, stained material) punctate basophilism²; iron granules.

Material and methods

Blood was obtained from samples from normal volunteers, or was taken from the residue of clinical samples submitted for "routine" blood counts. In all cases the anticoagulant was ethylenediaminetetraacetic acid (edetic acid) dipotassium salt. Pilot experiments showed that heparinised samples gave similar results.

Seminal fluid was from samples submitted for fertility testing.

Dyes used were Janus green B, colour index (CI) No 11050 from GT Gurr, specified "for mitochondrial staining", as an aqueous solution of 0.1% w/v; acridine orange (basic orange 14, CI No 46005) as a 0.1% w/v aqueous solution for vital staining, and as a 0.3% solution in citrate-saline for staining reticulocytes; brilliant cresyl blue CI No 51010, (BDH Poole, Dorset) 0.5% in ethanol (for reticulocytes); 1% in citrate saline, for unstable haemoglobin precipitation and staining.

Stains used were modified Wright's stain (Mercia Diagnostics); eosin BDH Poole Dorset. 1.0% aqueous; citrate-saline (trisodium citrate 3.8% in water 1 part; sodium chloride 0.85% in water 4 parts).

IMMERSION AND MOUNTING MEDIA

A set of Cargille Index of refraction liquids (RP Cargille Laboratories, Cedar Grove, New Jersey, USA) was used to study refraction effects. These were applied under a No 1 coverslip to prevent mixing with the immersion oil.

To conserve the expensive reference fluids, a medium of approximately defined refractive index ('n') was made by mixing dimethyl phthalate (n = 1.514 to 1.516) with dibutyl phthalate (n = 1.492 to 1.494), or with 1-Bromo-naphthalene (n = 1.6583 (BDH, Poole, Dorset). The resultant 'n' can be calculated from the formula:

$$n_m \times v_m = n_1 \times v_1 + n_2 \times v_2$$

where $v_{1&2}$ and $n_{1&2}$ are the volume and refractive index, respectively, of the components, and v_m and n_m of the mixture.

For permanent preparations either BPS³ or Euperal Vert (Difco) were used.

STAINING METHODS

Vital (non-lethal) staining was obtained with acridine orange and Janus green B: 5 μ l of 0.1% solution of dye is added to 0.5 ml of whole blood, mixed, and allowed to react for 20 to 60 minutes, then prepared for microscopy as films or buffy coat smears. Acridine orange may be combined with Janus green B as a counter stain, 5 μ l of each being added to 0.5 ml of blood.

Post-vital (lethal) staining was obtained with brilliant cresyl blue (for reticulocytes): 4 drops of a 0.5% solution of the dye in ethanol are dried in a 10 mm diameter plastic tube. Four drops of whole blood are added, left to stain for 10 minutes, and then smeared as thin films.

Brilliant cresyl blue was used for unstable haemoglobin precipitation and staining*: 5 drops of whole blood are added to an equal volume of a 1% solution of the dye in citrate-saline and allowed to stain for either two hours at 37°C or overnight at room temperature. Films are then made in the usual way.

Acridine orange was used for reticulocytes: one drop of 0.3% solution is mixed on a slide with one drop of blood and after 20 seconds smeared and dried.

Wright's stain for blood films was applied using the Ames Haematek staining machine. Perl's stain was used for iron. The film was fixed in methanol for 30 minutes, air dried, stained in: 4% hydrochloric acid (1 volume) 4% potassium ferrocyanide (5 volumes) for 10 minutes; rinsed in 1% lithium carbonate and in distilled water; counterstained in 1% aqueous eosin; washed and dried.

Thin blood films were made in the usual way.

MICROSCOPY

The smears were either unmounted, using Leitz immersion oil ($n = 1.518$) or mounted in one of a variety of media of known 'n' separated from the immersion oil by a No 1 coverslip. Oil immersion objectives (Zeiss $\times 40$ planapochromat Numerical Aperture (NA) 1.0 with iris diaphragm; Reichert planapochromat $\times 100$ NA 1.25 with iris diaphragm) were used, mounted on a Reichert Zetopan II microscope using Tungsten illumination and an oil immersion dark ground condenser NA 1.42.

A Leitz Ortholux II microscope with Ploem illuminator equipped for Rhodamine fluorescence was used.

APPLICATION OF IMMERSION/MOUNTING MEDIA

As dark ground microscopy is very sensitive to discontinuities in refractive index it is important to avoid air bubbles in the medium. Usually this can be achieved by wetting the smear with xylene before applying mountant or oil to the slide. The xylene seems to cause no optical disturbance.

Results

UNSTAINED MATERIAL

Plasmodia

Thin blood films containing *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium falciparum* were examined. Parasites showed as a cluster of highly refractile slightly bluish granules (fig 1). Bright field illumination showed that the granules were malarial pigment. *Plasmodium vivax* gave the weakest signal, although this may depend on the maturity of the trophozoites and hence the pigment content. Some distinguishing features of the type of plasmodia are: pigment granules in trophozoites of *P vivax* are finer than in other forms; the band form of trophozoite in *P malariae* may be recognised; applique forms of *P falciparum* are well shown.

In Giemsa stained "thick films" gametocyte pigment shows as clumps of brightly refractile granules which permits rapid screening, with verification under bright field illumination. Beck *et al* have shown similar effects during immunofluorescence studies on malaria.⁵

Another pigment, melanin, has an identical appearance under dark ground illumination (fig 4).

Some of the leucocytes can be identified in the unstained state. Neutrophil granulocytes are packed with tiny refractile granules of a slightly greenish hue, the nuclear lobes showing as negative staining among the granules. Eosinophils have coarser, highly refractile, slightly yellow, hollow granules.

Dark ground examination of unstained films can show inclusions in red cells. In films from cases of hyposplenism, of severe alcoholism, and from premature infants a variety of droplets, granules, and incrustations with iron can be seen (fig 3). Iron granules are very refractile and have pronounced greenish-blue colour. This identification was

Fig 1 Lymphocyte from a case of glandular fever showing ring-like structure of mitochondria. (Janus Green B, dark ground.)

Fig 2

Fig 3

Fig 4

Fig 5

Fig 6

Fig 7

Fig 2 Band form of *Plasmodium malariae*, (Unstained, dark ground.)

Fig 3 Red cells from chronic alcoholic showing inclusions and vacuoles. (Unstained, dark ground.)

Fig 4 Melanin granules in monocyte. Buffy coat from case of melanocarcinoma. (Wright's stain, dark ground.)

Fig 5 Mitochondria in platelets. Non-staining ferruginous granules in red cells. (Janus green B vital, dark ground.)

Fig 6 Monocytes (bottom left, top right and lymphocyte (centre) showing yellow mitochondria. Polymorphs (left and centre) showing mostly non-staining granules. (Janus green B, dark ground.)

Fig 7 Neutrophil showing rod-like mitochondria: red cells with solitary and multiple mitochondria. (Janus Green B, dark ground.)

confirmed by subsequent staining of marked fields by Perls's stain and examination under brightfield illumination. Under dark ground, Prussian blue granules are bright red against the vivid green of the eosin stained red cells.

VITALLY STAINED BLOOD

Janus green B

With this dye, mitochondria are seen as bright orange-yellow granules which, at the highest mag-

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nification, appear complex, consisting of signet-ring or bracelet-like groups of smaller components (fig 4). Granule-containing cells were identified by marking or charting fields under dark ground then staining the smears with Wright's stain for brightfield examination, or by mounting the smear in medium of slightly lower refractive index (n) to show the plasma and nuclear membranes of the cell. Euperal Vert ($n = 1.47$) is satisfactory and is a permanent mountant.

The granules are normally seen in a few red cells (less than 1.0%), in most platelets (fig 5), in two thirds of monocytes, in nearly all lymphocytes, in about 10% of neutrophils (fig 7), and rarely in eosinophils. Their size varies from 0.3 μm to 1.0 μm . They are largest in lymphocytes, especially activated forms, and smallest in monocytes and platelets. Monocytes contain the largest number of Janus green staining bodies (up to 100), lymphocytes up to 25, neutrophils up to seven, platelets from two to 25, eosinophils one or two. In granulocytes the Janus green bodies are often "bacillary" in form (fig 7). Many more Janus green bodies may be seen in neutrophils from cases showing neutrophil leucocytosis and toxic granulation.

In glandular fever, in samples showing many Turk cells, and in blood treated with phytohaemagglutinin the lymphocyte mitochondria seem larger and are more obviously annular (fig 1). In blood from infants Janus green staining of lymphocytes ranges from nil to the intense concentration seen in Turk cells. In chronic lymphocytic leukaemia the granules are more numerous (fig 8) and may be larger.

Janus green staining granules may be seen in up to 1% of red cells in normal blood. The proportion of such cells may be increased, both in cases showing reticulocytosis and when splenic function is diminished or absent. By combining vital Janus green staining and post-vital acridine orange staining, mitochondria and reticulocytes can be studied in the same preparation. Janus green is added to blood and after 15 to 30 minutes at 20°C a drop of the mixture is stained for reticulocytes with acridine orange. In addition to the orange-yellow mitochondrial granules, such preparations show nuclei of a dark dull green colour, and reticulocyte granules coloured a vivid turquoise. Other highly refractile pale turquoise granules are seen in all platelets and in some lymphocytes, red cells, monocytes and polymorphs (fig 9). Using this combined method, up to one third of reticulocytes show stained mitochondria, and nearly all red cells with stained mitochondria contain reticulocyte material, the exception being cases of hyposplenism, severe alcoholism, and prematurity in infants. In alcoholics as many as 17% of the red cells may have stained mitochondria unaccompanied by reticulocyte material—that is, in "mature" cells—

refractile white or pale turquoise granules also seen in the red cells in these cases look like defunct remnants of mitochondria.

When mouse blood containing trypanosomes (*T. Brucei*) was stained with Janus green in the usual way it showed intense staining of the kinetoplasts and less consistent, weaker staining of other granules along the bodies of the parasites. These less intensely stained granules showed best when the preparation was mounted in Euperal Vert (fig 10).

The combination of vital staining with Janus green B, quick drying, and dark ground microscopy can be applied to cells cultured on coverslips. Fibroblasts, normal keratinocytes, and squamous carcinoma cells⁶ were stained by adding Janus green B to the supernatant culture medium to a final concentration of 5×10^{-6} w/v. After 20 minutes the coverslips were rinsed in balanced salt solution containing 2% bovine albumin (to minimise crystallisation of salts during drying). This method shows that mitochondria are long beaded and thread-like in fibroblasts (fig 11), granular in keratinocytes, and apparently more abundant in the malignant cells (fig 12). The appearances are almost identical with those of cells vitally stained with the fluorochrome laser dye Rhodamine 123, reported by Johnson *et al.*⁷

When Janus green B is applied to seminal fluid in the same way as to blood, about one third of the spermatozoa show granular staining of the mitochondrial spiral. The association between staining and motility has not yet been established.

Acridine orange (vital staining)

This provides an excellent counterstain for mitochondrial staining with Janus green B and can be applied simultaneously. Cell granules show various shades of bluish-green and the nuclei have a more yellowish-green colour; basophil granules are brightly refractile and of an unsaturated turquoise colour.

Brilliant cresyl blue (post-vital)

Blood stained for reticulocytes and viewed under darkfield conditions shows the ribonuclease as bright apple green granules, with the usual reticular distribution.¹ Heinz bodies formed "in vivo" have an orange tint; those made in vitro by exposure to phenylhydrazine have a strong red colour.

Brilliant cresyl blue is also used as the standard dye for showing the presence of "unstable" haemoglobin in the red cells, by exposing the blood to the dye for about two hours. The haemoglobin H (HbH) granules fill the red cell and under dark ground are yellow to red in colour (fig 14). Even when scanty the affected cells are easy to identify because of the high contrast of the image. The staining of unstable haemoglobin fades in a few weeks unless mounted in BPS.

Fig 8

Fig 10

Fig 12

Fig 8 *Lymphocytes in chronic lymphocytic leukaemia with densely packed mitochondria. (Janus green B, dark ground.)*

Fig 9 *Red cells containing precipitated RNA (greenish blue) and mitochondria. (Janus green B, acridine orange post-vital, dark ground.)*

Fig 10 *T Brucei in mouse blood showing kinetoplasts and mitochondrial granules. (Janus green B, dark ground.)*

Fig 11 *Fibroblast from coverslip culture showing chains of mitochondria. (Janus green B, dark ground.)*

Fig 12 *Squamous carcinoma cells in culture showing many mitochondria. (Janus green B, dark ground.)*

Fig 9

Fig 11

Fig 14

Fig 14 *Red cells, of which all but three are packed with precipitated HbH. One (lower centre) also contains highly refractile reticulocyte granules. (Brilliant cresyl blue, post-vital, dark ground.)*

Nizet used dyes other than cresyl blue for the identification of reticulocytes under darkfield microscopy, and of these the best is acridine orange; staining is almost instantaneous, and the colour (saturated turquoise) is as vivid as when the same dye is used as a fluorochrome.

Wright's stain

Under darkfield illumination Romanowsky stained preparations show enhanced detail of nuclear struc-

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ture and, by refraction, show cytoplasmic granules that are poorly visualised under brightfield (fig 2). Dark ground is especially useful for showing punctate basophilism and was formerly used in screening for lead poisoning.²

Discussion

Dark ground microscopy has largely been neglected in haematology, perhaps because it has the reputation for being an exacting method, but the modern "research" microscope is usually equipped with a multipurpose substage system which avoids most of the difficulty in setting up. The staining techniques we describe are simple to apply, mostly requiring no more than the addition of a few microlitres of dye to the blood sample.

The colours displayed by the stained granules were at first surprising, in view of the colour of the same objects seen in transmitted light. Reference to spectral data for the dyes used⁸ shows that the absorption peaks for the dyes correspond to the colours seen under darkfield conditions. This can be verified by placing a calibrated graded interference filter in the narrow light beam coming from the substage of the microscope. By moving the filter to and fro, the wavelength of maximum granule brightness can be identified and confirmed to be that of the spectral absorption peak for the dye. It seems that the dyed granule acts as a dichroic mirror, passing those wavelengths we see in brightfield, reflecting those seen in the dark ground.

The reflection colour is modified by admixture with light transmitted through the granules and by reaching the objective as a result of refraction. Thus the colour and contrast of the image can be modified by changing the refractive index of the mounting medium which changes the amount of refracted light. Further variation of the reflection signal seems to result from the nature of the surface to which the dye is attached. For instance, brilliant cresyl blue stains platelet granules greenish yellow and the released lipid dark red.

Until the introduction of electron microscopy for the study of cells, the standard method of identifying mitochondria was by vital staining with Janus green B⁹ or Pinacyanol.¹⁰ Since then the method has been little used, probably because in the standard technique (using wet preparations under brightfield illumination) contrast is low and the granules and cells are in constant Brownian movement. The combination of methods described here gives images of high contrast from stable, permanent preparations. The ultrastructural studies of Tanaka¹¹ confirm that vital staining with Janus green B in low concentration is specific for mitochondria. Electron micrographs of lymphocytes stained with Janus green B show

Fig 13 Mitochondria in phytohaemagglutinin-stimulated lymphocytes: (a) unstained. (b) after Janus green B. Note myelin figure formation and dye precipitation in mitochondria.

extensive damage to mitochondria (fig 13), suggesting that the dye is more toxic than formerly supposed.¹² Electron microscopy shows one major discrepancy; many more mitochondria are detected in granulocytes than by the dye method. When the fluorochrome Rhodamine 6G is used instead of Janus green B, many mitochondria are seen in the granulocytes, which agrees with the ultrastructural findings. For all other blood cells, Janus green B, Rhodamine 6G, and electron microscopy seem to give comparable results.

The reason for the discrepancy may lie in the variable plasma membrane permeability of the different cell types for each dye used. Granulocytes from cases showing a toxic neutrophil leucocytosis and increased Janus green B staining, may have plasma membranes that are more permeable by the dye.

The other dye classically used for mitochondrial staining is Pinacyanol. It gives vivid staining but has a low solubility in aqueous media and the colour fades.

The use of dark ground illumination greatly enhances the contrast of unstable haemoglobin precipitated by brilliant cresyl blue. Experiments now

in progress suggest that certain combinations of dye concentration, time, temperature and age of sample may give false positive reactions. The increased sensitivity of the dark ground method should permit better standardisation of this test.

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