A RAPID METHOD FOR THE DIFFERENTIAL STAINING OF BLOOD FILMS AND MALARIAL PARASITES.

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Since Romanowsky, in 1891, described a method of staining the malarial parasites by which the chromatin and cytoplasm were differentially stained, various workers have brought forward modifications of his method, designed to overcome its uncertainties and to simplify it.

All of these methods, with the exception of that of Leishman (British Medical Journal, Sept. 21, 1901), have been so complicated, or so time-consuming, or so inconstant in results, or have offered so many difficulties and uncertainties in the preparation of the staining fluid, that they cannot be regarded as practical methods to be used by any but the more skillful workers.

The following method is a simplification of Leishman's method, which latter is a modification of the well-known methylic alcohol method of Jenner for staining blood films. As in Jenner's method, the troublesome fixation of the blood by heat, which is the greatest difficulty in the way of obtaining good preparations of blood by the common methods of staining, is done away with. The method gives not only a differential staining for the chromatin and the cytoplasm of malarial parasites, but it is also a general staining method for blood and as such is superior to Jenner's method, in that it sharply differentiates the nuclei and the cytoplasm of the lymphocytes and mononuclear leucocytes, as well as the granulations of the leucocytes in general.

It is confidently believed that by following out with reasonable accuracy the procedures detailed below, this simple method will not fail to give constantly the results promised for it, even in the hands of beginners in hematology.

The method is as follows:

Preparation of the Staining Fluid.

Make a one-half per cent solution of sodium bicarbonate in water in an Ehrlenmeyer flask and add to it one per cent of methylene blue (Grübler). I have found that any of the methylene blues of Grübler known as "BX," "Koch's," or "Erlich's Rectified" may be used. It seems to be important that the bicarbonate of soda be all dissolved before adding the methylene blue.

The mixture is next to be steamed in an Arnold steam sterilizer for one hour, counting the time after "steam is up." This steaming of the alkaline solution of methylene blue effects certain changes in the methylene blue whereby a poly-chromatic property is given to it, so that the compound with eosin, which is later to be formed with it, has the property not only of differentially staining the chromatin of the malarial parasite, but also of differentiating and bringing out more sharply the nuclei and granules of the white blood corpuscles. That the methylene blue must be modified in order to produce the Romanowsky stain in the malarial parasite has been generally known and the modification has been brought about by different writers in various ways, all of which either require too much time or are too difficult of execution to be satisfactory to ordinary laboratory workers.

When the steaming is completed, the mixture is removed from the sterilizer and allowed to cool, the flask being placed in cold water if desired. When it is cold, without filtering, pour it into a large dish or flask and add to it, stirring or shaking meanwhile a sufficient quantity of a one to one thousand solution of eosin (Grübler, yellowish, soluble in water) until the mixture, losing its blue color, becomes purple in color, and a scum with yellowish metallic luster forms on the surface, while on close inspection a finely-granular black precipitate appears in suspension. This will require about five hundred cubic centimeters of the eosin solution for one hundred cubic centimeters of the alkaline methylene blue solution. These are quantities which are convenient and suitable to employ.

The precipitate is collected on a filter and without washing is allowed to dry thereon. When thoroughly dry a saturated solution in pure methylic alcohol is made. Three-tenths of a gramme of the dry precipitate will thoroughly saturate one hundred cubic centimeters of the methylic alcohol in a few minutes.

The saturated alcoholic solution of the precipitate is next filtered and to the filtrate is then added twenty-five per cent of methylic alcohol; *e.g.*, to eighty cubic centimeters of the saturated alcoholic solution twenty cubic centimeters of methylic alcohol is added.

This somewhat diluted alcoholic solution of the precipitate is the staining fluid. It is permanent and may be kept on hand ready for use. Care should be taken to prevent the alcohol from evaporating and thus making the solution too saturated. The object of diluting the saturated solution is to prevent precipitations on the blood film in the process of staining.

The Staining of the Blood Film.

The films of blood, which should be spread thinly, are allowed to dry in the air. When dry, as much of the staining fluid is poured upon a film as the cover glass or slide will readily hold without draining off. Allow the staining fluid to remain in contact with the film for one minute. This chiefly serves the purpose of fixing the blood corpuscles. If the film is spread on a cover glass, the cover glass is most conveniently manipulated by means of cover-glass forceps.

Next add to the staining fluid on the cover glass or slide sufficient water, drop by drop, until it becomes semi-translucent and a reddish tint becomes visible at its margins, while a metallic scum forms on the surface. The amount of water required will vary with the amount of staining fluid on the preparation, but in general it may be said that eight or ten drops will be required if a seven-eighth inch square cover glass is used.

The staining fluid, thus diluted, is allowed to remain on the preparation for two or three minutes, during which time

the real staining of the preparation takes place, and is then washed in water.

The blood film will now be seen to have a blue or purple color, and if examined with the microscope the red blood corpuscles will be seen to be stained blue.

The next step is to develop the differential staining of the various elements in the preparation. This is done by washing the preparation in water, preferably distilled water, until the better spread portions of the film appear yellowish or reddish in color. If desired, the process of differentiation may be readily observed by placing the cover glass film-side uppermost on a slide, covering it with water, and examining it with the microscope under a low magnifying power. The red blood corpuscles, which as before stated at first have a blue color, will become greenish, then yellowish, and finally orange or pinkish in color, depending upon the depth of the original staining, which varies with the length of time that the diluted staining fluid has been allowed to act, and with the degree of its dilution.

This differentiation by washing in water seems to be essentially a process of decolorization by which some of the blue constituent of the dye is removed, for the water that drains off from the preparation has a blue color. This differentiation or decolorization proceeds slowly, and may require one to three minutes, depending upon the intensity of the staining, and upon the tint sought to be obtained in the red corpuscles.

It is apparent from the above that with a little experience with the method the color of the red corpuscles may be made either orange or pink, as the operator desires. When the desired color is obtained in the red corpuscles the preparation is then quickly dried between layers of filter paper and mounted in balsam. It is important to stop the decolorization by drying the preparation as soon as the desired tint in the red corpuscles is obtained, for it may be carried too far.

In the light of the foregoing explanations, the following summary of the method of staining blood films will be intelligible:

1. Make films of the blood, spread thinly, and allow them to dry in the air.

2. Cover the preparation with the alcoholic solution of the dye for one minute.

3. Add to the alcoholic solution of the dye on the preparation sufficient water, drop by drop, until the mixture becomes semi-translucent, and a yellowish metallic scum forms on the surface. Allow this mixture to remain on the preparation for two or three minutes.

4. Wash in water, preferably in distilled water, until the film has a yellowish or pinkish tint in its thinner or better spread portions.

5. Dry between filter paper and mount in balsam.

Dried blood films may be kept for some weeks without impairment of their staining properties. Films months old will probably not give good results.

Microscopical Appearances in Blood Films stained by this Method.

The *red cells* are orange or pink in color. Polychromatophilia and punctate basophilia (the granular degeneration of Grawitz) are well brought out. The nucleated red cells have deep blue nuclei and the cytoplasm is usually of a bluish tint.

The *lymphocytes* have dark purplish-blue nuclei and robin's egg blue cytoplasm in which a few dark blue or purplish granules are sometimes present.

The *polynuclear neutrophilic leucocytes* have a dark blue or dark lilac colored nucleus, and the granules are usually of a reddish lilac color.

The *eosinophilic leucocytes* have blue or dark lilac colored nuclei. The granules have the color of eosin, while the cytoplasm in which they are imbedded has a blue color.

The *large mononuclear leucocytes* appear in at least two forms. Each form has a blue or dark lilac colored nucleus. The cytoplasm of one form is pale blue and of the other form is blue with dark lilac or deep purple colored granules, which are usually not so numerous as are the granules in the polynuclear neutrophilic leucocytes.

The *Mast cells* appear as cells of about the size of polynuclear leucocytes with purplish or dark blue stained, irregular-shaped nuclei, and cytoplasm, sometimes bluish, in which numerous coarse spherical granules of variable size are imbedded. These granules are of a dark blue or of a dark purple color, and may appear almost black.

The *myelocytes* have dark blue or dark lilac colored nuclei and blue cytoplasm in which numerous dark lilac or reddish lilac colored granules are imbedded. In leukemia more color differences are brought out among the leucocytes than by the ordinary methods of staining.

The *blood plates* are deeply stained and are a prominent feature of nearly every blood preparation. They appear as blue or purplish rounded or oval bodies, usually of a diameter of one-third to one-half of that of a red blood corpuscle. They have ragged margins and present fine blue or purplish dots or mottlings in their substance. Sometimes they appear in groups or masses, and at first sight may be regarded as precipitates. In many instances they have the appearance of being within a red corpuscle and surrounded by an unstained zone of its cytoplasm. Occasionally a blood plate presents the appearance of being partly inside and partly outside the body of a red blood corpuscle.

While these appearances may be artefacts or due to the adhesion of blood plates to the surface of red corpuscles, their occurrence is sufficiently frequent to suggest the idea of the possibility that the blood plates represent the degenerated and extruded chromatin remains of the nuclei of the red blood corpuscles. The blood plates apparently situated inside the red cells are very likely to be mistaken by unpractised observers for young malarial parasites. This point will be referred to below.

Malarial Parasites.

The body of a malarial parasite stains blue, while the color of the chromatin varies from a lilac color through varying shades of red to almost black. In the young forms of the tertian and estivo-autumnal parasites the chromatin appears

as a spherical very dark red body, while in the older forms of the tertian parasite it has a more lilac or purplish red color and may appear in the form of a reticulum. In the intermediate forms the color of the chromatin may present variations between these extremes. In preparations examined in water mounting instead of balsam the distinct red color of the chromatin is more apparent than in the preparations mounted in balsam.

As above mentioned, the blood plates apparently situated within red blood corpuscles may be mistaken by the inexperienced for young malarial parasites. This ought never to occur if one bears in mind the fact that the young parasite of all of the three kinds should present by this method a dark red spherical nucleus and a cytoplasm which is usually in the form of a definite ring.

For those who are not familiar with the morphology of the malarial parasites, a study of the excellent descriptions and plates of the same contained in Ewing's "Clinical Pathology of the Blood" is recommended.

Various workers have shown by their modifications of the Romanowsky method that red blood corpuscles harboring malarial parasites have dark red staining granules. These granules may be brought out by the present method, but in order to bring them out it is necessary to allow the staining fluid, after the addition of the water to it, to remain on the preparation for at least five minutes and then not to decolorize or differentiate with water for as long a time or to such an extent as for ordinary blood preparations.

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