CHAPTER 1

Blood sampling and blood film preparation and examination

Obtaining a blood specimen

Performing an accurate blood count and correctly interpreting a blood film require that an appropriate specimen from the patient, mixed with the correct amount of a suitable anticoagulant, is delivered to the laboratory without undue delay. No artefacts should be introduced during these procedures.

The identity of the patient requiring blood sampling should be carefully checked before performing a venepuncture. This is usually done by requesting the patient to state surname, given name and date of birth and, for hospital inpatients, by checking a wristband to verify these details and, in addition, the hospital number or other unique identifying number. To reduce the chance of human error, bottles should not be labelled in advance. The person performing the phlebotomy must conform to local guidelines, including those for patient identification. Although traditionally more attention has been given to patient identification in relation to blood transfusion, it should be noted that wrong treatment has also followed the misidentification of patients from whom samples are taken for a blood count and identification must also be taken seriously in this field. More secure identification of inpatients can be achieved by the use of electronic devices, for example by scanning the patient's identity on a bar-coded wristband by means of a hand-held device.

Patients should either sit or lie comfortably and should be reassured that the procedure causes only minimal discomfort; they should not be told that venepuncture is painless, since this is not so. It is best for apprehensive patients to lie down. Chairs used for venepuncture should preferably have adjustable armrests so that the arm can be carefully positioned. Armrests also help to ensure patient safety, since they make it harder for a fainting patient to fall from the chair. I have personally observed one patient who sustained a skull fracture when he fainted at the end of a venepuncture and fell forward onto a hard floor, and two other patients, neither previously known to be epileptic, who suffered epileptiform convulsions during venepuncture. Such seizures may not be true epilepsy, but consequent on hypoxia following brief vagal-induced cessation of heart beat [1]. If venepuncture is being performed on a child or on a patient unable to cooperate fully, then the arm for venepuncture should be gently but firmly immobilised by an assistant. Gloves should be worn during venepuncture, for the protection of the person carrying out the procedure as well as the patient. Hands should be washed before putting on gloves and again after gloves are removed. Non-latex gloves must be available if either the phlebotomist or the patient is allergic to latex. The needle that will enter the patient's vein must not be touched so that it remains sterile.

In some circumstances, the patient should rest prior to venesection. In endurance athletes being tested for a 'biological passport', 10 minutes' rest in a seated position has been found to be sufficient for the haemoglobin concentration (Hb) and haematocrit (Hct) to fall to a stable level [2].

Peripheral venous blood

In an adult, peripheral venous blood is most easily obtained from a vein in the antecubital fossa (Fig. 1.1) using a needle and either a syringe or an evacuated tube. Of the veins in the antecubital region, the median cubital vein is preferred since it is usually large and well anchored in tissues, but the cephalic and basilic veins are also often satisfactory. Other forearm veins can be used, but they are frequently more mobile and therefore more difficult to penetrate. Veins on the dorsum of the wrist and hand often have a poorer flow and performing venepuncture at these sites is more likely to lead to bruising. This is also true of the anterior surface of the wrist where, in addition, venepuncture tends to be more painful and there is more risk of damaging vital structures. Foot veins are not an ideal site for venepuncture and it is rarely necessary to use them. Injuries that have been

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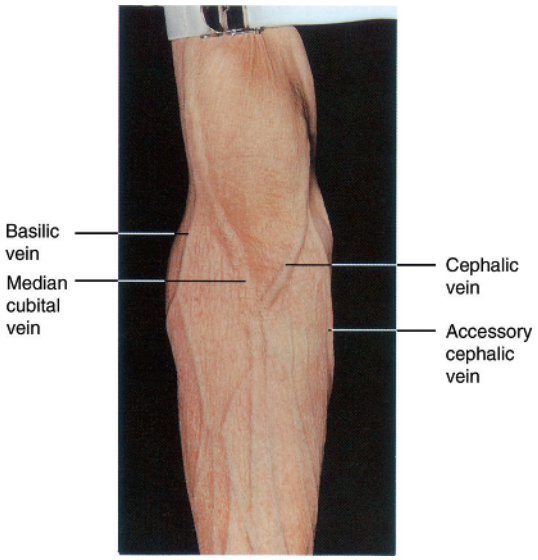

Fig. 1.1 Anterior surface of the left arm showing veins most suitable for venepuncture.

associated with obtaining a blood sample from the antecubital fossa include damage to the lateral antebrachial cutaneous nerve [3] and inadvertent arterial puncture. Complications are more likely with the less accessible basilic vein than with the median antecubital or the cephalic vein. If anterior wrist veins have to be used, there is a risk of damage to the radial or ulnar nerve or artery. Use of foot veins is more likely to lead to complications, such as thrombosis, infection or poor healing.

When a vein has been identified, it is palpated to ensure it is patent. A patent vein is soft and can be compressed easily. A thrombosed vein feels cord-like and is not compressible. An artery has a thicker wall and is pulsatile. If a vein is not visible (in some dark-skinned or overweight people) it can be identified by palpation after applying a tourniquet to achieve venous distension. If the veins appear to be very small, warming of the arm to produce vasodilatation or tapping the vein and asking the patient to clench and unclench the fist several times may help.

It should be noted that pathogenic bacteria can be cultured from reusable tourniquets and it is prudent practice to use disposable tourniquets, at least for patients at particular risk of infection [4].

The patient's arm should be positioned on the armrest so that the vein identified is under some tension and its mobility is reduced. The skin should be cleaned with 70% ethanol or 0.5% chlorhexidine and allowed to dry, to avoid stinging when the skin is penetrated. A tourniquet is applied to the arm, sufficiently tightly to distend the vein, but not so tightly that discomfort is caused. Alternatively, a sphygmomanometer cuff can be applied and inflated to diastolic pressure, but the use of a tourniquet is usually quicker and simpler. If it is particularly important to obtain a specimen without causing haemoconcentration, for example in a patient with suspected polycythaemia, the tourniquet should be left on the arm only long enough to allow penetration of the vein. Otherwise it can be left applied while blood is being obtained, to ensure a continuing adequate flow of blood. It is preferable that the tourniquet is applied for no more than a minute, but the degree of haemoconcentration may not be great, even after 10 minutes' application. In one study the increase of the Hb and the red blood cell count (RBC), usually referred to simply as the red cell count, was about 2% both at 2 and at 10 minutes [5]. However, in another study Hb rose by 9 g/l by 3 minutes and RBC and Hct by a corresponding amount [6].

Blood specimens can be obtained with a needle and an evacuated tube (see below) or with either a needle or a winged blood collection cannula (a 'butterfly') and a syringe. A winged cannula may reduce the chance of injury to nerves [7] and is certainly preferable for small veins and difficult sites. A 19 or 20 gauge needle is suitable for an adult and a 21 or 23 gauge for a child or an adult with small veins. When using a syringe, the plunger should first be moved within the barrel of the syringe to ensure that it will move freely. Next the needle is attached to the syringe, which, unless small, should have a side port rather than a central port. The guard is then removed. The needle is now inserted into the vein with the bevel facing upwards (Fig. 1.2). This may be done in a single movement or in two separate movements for the skin and the vein, depending on personal preference and on how superficial the vein is. With one hand steadying the barrel of the syringe so that the needle is not accidentally withdrawn from the vein, blood is withdrawn into the syringe using minimal negative pressure. Care should be taken not to aspirate more rapidly than blood is entering the vein, or the wall of the vein may be drawn against the bevel of the needle and cut off the flow of blood. If the tourniquet has not already been released, this must be done before withdrawing the needle. Following removal of the needle, direct pressure is applied to the puncture site with cotton wool or a sterile gauze square, the arm being kept straight and, if preferred, somewhat elevated. Adhesive plaster should not be applied until pressure has been sustained for long enough for bleeding from the puncture site to have stopped.

The needle should be removed from the syringe before expelling the blood into the specimen container, great care being taken to avoid self-injury with the needle. The needle should

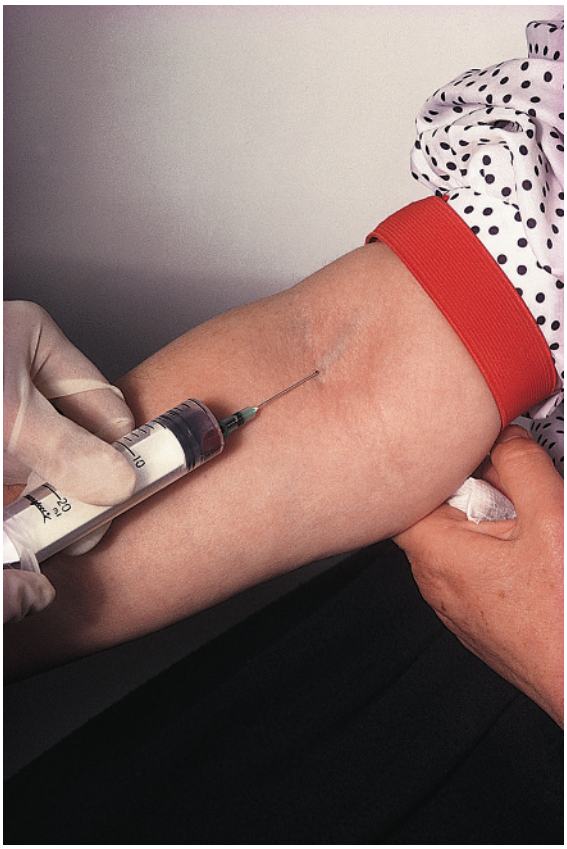

Fig. 1.2 Venepuncture technique using needle and syringe.

be put directly into a special receptacle for sharp objects without resheathing it, unless the needle incorporates a special device that can be flicked into place without risk to the fingers. The blood specimen is expelled gently into a bottle containing anticoagulant and is mixed gently by inverting the container four or five times. Forceful ejection of the blood can cause lysis. Shaking should also be avoided. The specimen container is then labelled with the patient's name and identifying details and, depending on the hospital's standard operating procedure, possibly also with a barcode label, which is also applied to the request form and subsequently to the blood film. The time of venepuncture should also be recorded on the bottle. Bottles should not be labelled in advance away from the patient's bedside as this increases the chances of putting a blood sample into a mislabelled bottle. Recording the time of venepuncture is important both to allow the clinician to relate the laboratory result to the condition of the patient at the time and also to allow the laboratory to check that there has been no undue delay between venepuncture and performing the test.

Evacuated tube systems include Vacutainer (BD) and Vacuette (Greiner Bio-One). When blood is taken into an evacuated tube the technique of venepuncture is basically similar to that described above. A double-ended needle is screwed into a holder, which allows it to be manipulated for venepuncture (Fig. 1.3). Alternatively, a winged cannula can be attached to an evacuated tube, using a plastic holder into which an adaptor is screwed. Once the vein has been entered, an evacuated tube is inserted into the holder and is pushed firmly so that its rubber cap is penetrated by the needle, breaking the vacuum and causing blood to be aspirated into the tube (Fig. 1.4). Evacuated tubes are very convenient if multiple specimens are to be taken, since several evacuated tubes can be applied in turn. Only sterile vacuum tubes should be used for obtaining blood specimens. In children or adults with very small veins, an appropriately small vacuum tube should be used so that excessive pressure does not cause

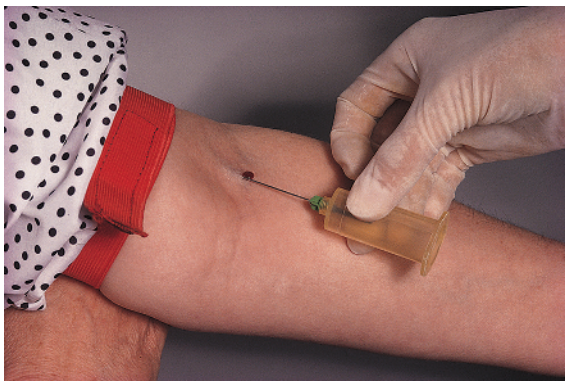

Fig. 1.3 Venepuncture technique using an evacuated container. The distal end of the needle has been screwed into the holder; the proximal end has then been unsheathed and inserted into a suitable vein.

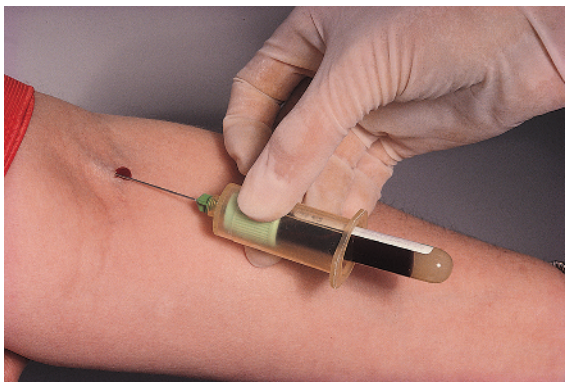

Fig. 1.4 Venepuncture technique using an evacuated container. The evacuated container has been inserted into the holder and forced onto the end of the needle.

Table 1.1 Clinical and Laboratory Standards Institute recommended order for taking blood samples [8].

Blood culture tubes
Sodium citrate tubes
Plain tubes or gel separator tubes for serum
Heparin tubes/heparin gel separator tubes
EDTA tubes
Fluoride tubes for glucose

EDTA, ethylenediaminetetra-acetic acid.

the vein to collapse. Once all necessary specimen tubes have been filled, the needle is withdrawn from the vein, still attached to the holder. To reduce the possibility of a needle-prick injury it is necessary to either: (i) use a specially designed device that permits the needle to be discarded with a single-hand technique; (ii) remove the needle from the holder with a specially designed safe device; or (iii) throw away the holder with the needle. The tubes of blood should be mixed promptly. When blood samples are obtained with an evacuated tube system the anticoagulant from one tube may contaminate the next one used. Heparin may interfere with coagulation tests, ethylenediaminetetra-acetic acid (EDTA) with calcium measurements and fluoride with haematological investigations. It is therefore advised that samples be taken in the order shown in Table 1.1.

If there is a need for a large specimen or a large number of specimens, either an evacuated tube system or a syringe and winged cannula should be used. In the latter case the tubing is pinched off to allow several syringes in turn to be attached. This technique is also useful in children and when small veins make venepuncture difficult. If it is necessary to take blood from a small vein of the hand it may be advantageous to introduce a butterfly against the direction of the blood flow and use a syringe with gentle suction rather than an evacuated tube [9]. A blood specimen should not be taken from a vein above the site of an intravenous infusion, since dilution can occur. However, venepuncture below the site of an infusion is not associated with clinically significant inaccuracy.

'Capillary' blood

It is often necessary to obtain blood by skin puncture in babies and infants and in adults with poor veins. 'Capillary' or, more probably, largely arteriolar blood may be obtained from a freely flowing stab wound made with a sterile lancet on the plantar surface of a warmed and cleansed heel (babies less than 3 months of age and infants), the plantar aspect of the big toe (infants) or a finger, thumb or ear lobe (older children and adults). The correct site for puncture of the heel

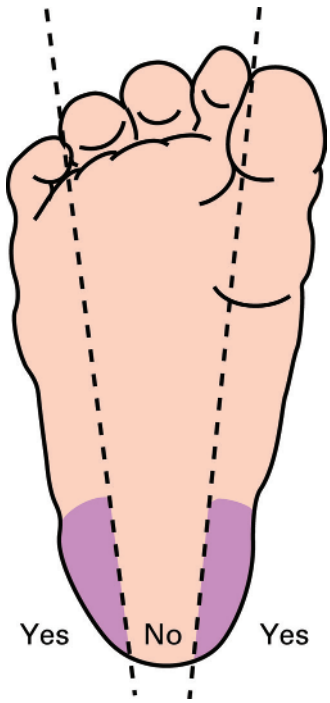

Fig. 1.5 The areas of the foot of a baby or infant that are suitable for obtaining capillary blood.

is shown in Fig. 1.5. In a baby, the lateral or posterior aspect of the heel should not be used because the underlying bone is much closer to the skin surface than it is on the plantar aspect. In older patients a finger (excluding the fifth finger) or thumb is preferred to an ear lobe, since bleeding from the ear lobe may be prolonged in a patient with a haemostatic defect, and pressure is difficult to apply. The palmar surface of the distal phalanx is the preferred site on a digit, since the underlying bone is closer to the skin surface on other aspects. The middle or ring finger of the non-dominant hand is preferred; puncture of these digits is less painful than puncture of the index finger. In adults, skin punctures should ideally be more than 1.5 mm deep in order to ensure that the lancet passes through the dermal–subcutaneous junction where the concentration of blood vessels is greatest, permitting a free flow of blood.

Lancets used for heel puncture in full-term babies must not exceed 2.4 mm in length, since this is the depth below the skin of the calcaneal bone. Much shorter lancets are available and should be selected for use in premature babies. An automated lancet is preferred, the precise device being selected according to the weight of the baby. The maximum depth of penetration with an automated lancet is such that the centre of the plantar surface of the heel can also be used,

at least in term and late preterm babies [10]. Osteomyelitis of the calcaneal bone has resulted from inadvertent puncture of the bone [11]. Previous puncture sites should be avoided to reduce the risk of infection. Heel-prick sampling can lead to cutaneous calcification, which is harmless but can persist for years [12]. Safety lancets, with a blade that retracts permanently after first use, have been developed in order to reduce the risk of accidental injury to phlebotomy staff. They are available in sizes appropriate for adults, children, infants and premature neonates.

Capillary samples should be obtained from warm tissues so that a free flow of blood is more readily obtained. If the area is cool then it should be warmed with a wet cloth, no hotter than 42°C. The skin should then be cleansed with 70% isopropanol and dried with a sterile gauze square (since traces of alcohol may lead to haemolysis of the specimen). Because the first drop of blood may be diluted with tissue fluid, it should be wiped away with a sterile gauze square. Flow of blood may be promoted by gentle pressure, but a massaging or pumping action should not be used, since this may lead to tissue fluid being mixed with the blood.

Capillary blood can be collected into glass capillary tubes. These can be coated with EDTA, but tubes containing heparin are not suitable for full blood count (FBC) specimens since cellular morphology and staining characteristics are altered. Disposable pipettes complete with diluent, suitable for both automated and manual counts, are commercially available. Caution is necessary if glass capillary tubes are used, because of the risk of injury to the person obtaining the blood sample [13]. Caution should also be employed in the use of spring-loaded skin-prick devices, since transmission of hepatitis B from one patient to another has occurred when there has been a failure to change the platform as well as the lancet between patients [14]. However, automated lancets do ensure a standardised depth of penetration. Use of one proprietary automated incision device (Tenderfoot, Accriva Diagnostics) has been reported to cause less bruising and to be associated with less haemolysis of capillary samples than when the device is not used [15, 16].

Platelet counts performed on capillary blood are often lower than those on venous blood [17] and other parameters may also vary (see Chapter 5). The precision of Hb measurements on a single drop of capillary blood is poor and it is therefore recommended that several drops be put into an EDTA-containing tube [18].

The recommended order for taking capillary samples differs from that shown in Table 1.1 for venous samples. For capillary samples, because of the tendency of platelets to aggregate at the site of puncture, only a sample for blood gas analysis takes precedence over an EDTA sample for an FBC.

Cord blood

Blood samples can be obtained from the umbilical cord immediately after birth. Cord blood is best obtained with a syringe and needle after removing any blood from the surface of the cord with a gauze square. Expressing blood from the cut end of the cord can introduce Wharton's jelly into the blood sample, with subsequent red cell agglutination. Haematological parameters on cord blood are not necessarily the same as those obtained from capillary or venous specimens from the neonate.

Fetal blood

Blood samples can be obtained from a fetus by cordocentesis and can be diagnostically useful. A blood count and film can be useful not only when a haematological disorder is suspected, but also when a fetus is being investigated because of dysmorphic features detected on ultrasound examination [19].

Blood specimens from other sites

It may sometimes be necessary to obtain blood from the femoral vein or from indwelling cannulae in various sites. When blood is obtained from a cannula, the first blood obtained may be diluted by infusion fluid or contaminated with heparin and should be discarded. In infants, blood can be obtained from scalp veins or jugular veins.

Anticoagulants and specimen containers

The anticoagulant of choice for blood count specimens is one of the salts of EDTA. K_2EDTA, K_3EDTA and Na_2EDTA have all been used. The preferred anticoagulant, recommended by the International Council (then Committee) for Standardization in Haematology (ICSH), is K_2EDTA in a final concentration of 1.5–2.2 mg/ml [20]. Both dry EDTA and EDTA in solution are in use. If screw-capped tubes are being used, a solution has the advantage that mixing of blood specimens is easier, so clotted specimens are less common. However, if a dry evacuated tube system is used, in which the inside of the tube is coated with the anticoagulant, poor mixing is not a problem. It should also be noted that some parameters are altered by dilution and, if too little blood is taken into a tube, dilution may be appreciable. Excess EDTA also has deleterious effects on cell morphology in stained blood films. Na_2EDTA is less soluble than the potassium salts. K_3EDTA causes undesirable cell shrinkage, which is reflected in a lower microhaematocrit.

Most laboratories use automated blood counting instruments with a sampling device that is able to perforate the rubber cap of a blood specimen container, thus reducing unnecessary handling of blood. To take advantage of this it is

necessary that not only evacuated tubes but also all blood containers have rubber caps that can be penetrated and resealed without permitting leakage.

Guidelines

Guidelines for the procedure of venepuncture [8] and for the protection of phlebotomists and laboratory workers from biological hazards [21] have been published by the Clinical & Laboratory Standards Institute (CLSI). It is recommended that 'standard precautions' proposed by the Centers for Disease Control and Prevention (CDC), previously referred to as 'universal precautions', be applied to phlebotomy. This policy means that all blood specimens are regarded as potentially infectious. The following specific recommendations are made [21]:

- Gloves should preferably be worn for all phlebotomy; their use is particularly important if the phlebotomist has any breaks in the skin, if the patient is likely to be uncooperative, if the phlebotomist is inexperienced or if blood is being obtained by skin puncture.
- Gloves should be changed between patients.
- An evacuated tube system should be used in preference to a needle and syringe.
- If a needle and syringe have to be used and it is then necessary to transfer blood to an evacuated tube, the rubber stopper should not be removed. The stopper should be pierced by the needle and blood allowed to flow into the tube under the influence of the vacuum. To avoid the possibility of a self-inflicted wound the evacuated tube **must not be held in the hand** during this procedure, but instead should be placed in a rack.

Needle-stick injury

Precautions should be taken to avoid needle-stick (needle-prick) injuries. Hepatitis B can be readily transmitted by such injury, particularly when the patient is hepatitis B e antigen positive. Overall transmission rates of 7–30% have been reported following needle-prick injuries involving infected patients. If the patient is e antigen positive, the rate of transmission is of the order of 20% if hepatitis B immunoglobulin is given after the injury and about 30–40% if it is not given [22, 23]. Reported rates of transmission of hepatitis C have varied from 0% to 7%, with a mean of 1.8% [24]; however, when sensitive techniques are used, the rate of transmission has been found to be about 10% [25]. Transmission occurs only from patients who are positive for hepatitis C viral RNA [24]. Human immunodeficiency virus (HIV) is much less readily transmitted than hepatitis B or C, but a risk does exist. In 3430 needle-prick injuries reported up to 1993 the overall transmission rate was 0.46% [26]. Other infections that have been transmitted occasionally by needle-prick injury include malaria, cryptococcosis, tuberculosis, viral haemorrhagic fevers (including Ebola virus), human T-cell lymphotropic virus 1 and 2 and dengue fever [27–32]. Public Health England Advisory Committee on Dangerous Pathogens guidance should be followed when blood tests are required in patients with suspected viral haemorrhagic fever [33].

A risk of injury and viral transmission also exists if glass capillary tubes are used for blood collection, and alternative blood collection devices have therefore been advised in the United States by the Food and Drug Administration (FDA) [13].

Because it has proved impossible to eliminate needle-stick injuries, all hospitals should have agreed policies for dealing with them. Both laboratory managers and occupational health services have a responsibility in this regard. Staff who suffer such injuries should be referred promptly to occupational health for documentation, baseline testing and management. Up-to-date protocols must be followed. Unless all new staff are routinely tested for HIV, occupational health services should consider at least offering storage of serum samples so that baseline HIV testing is possible in the event of a subsequent needle-prick injury. If this policy is not followed, serum storage should be offered in the event of a needle-prick injury from a seropositive source or from a source of unknown HIV status.

All staff who are performing venepunctures should be offered vaccination against hepatitis B and the adequacy of their antibody response should be verified; if a needle-prick injury from a known hepatitis B-positive source occurs, the antibody titre should be checked and a booster vaccination given if necessary [21]. Phlebotomists with an inadequate antibody response to vaccination should, in the event of a needle-prick injury from an infected source, be offered hepatitis B immunoglobulin. Phlebotomists who have chosen not to be vaccinated should be offered hepatitis B immunoglobulin and vaccination should again be offered.

Antiretroviral prophylaxis should be offered to those exposed to a known or possible risk of HIV exposure through needle-stick injury and ideally this should be administered within a few hours of exposure, while awaiting test results on the patient; the risk of infection becoming established is reduced but not eliminated by antiretroviral prophylaxis [34]. Multiagent antiretroviral therapy is now recommended. UK guidelines are available and are summarised by Riddell *et al*. [32]. The use of nevirapine is not recommended because of the possibility of serious toxicity [35].

There appears to be no effective post-exposure prophylaxis for hepatitis C infection [24], but the consensus view is that interferon or other therapy is indicated in acute infection,

including that acquired by needle-stick injury. Interferon alpha-2b in a dose of 5 million units daily for 4 weeks followed by the same dose three times a week for a further 20 weeks has been found to be efficacious [36]. Other treatment options include interferon plus ribavirin and combined direct-acting antiviral drugs.

Specimen mixing

The blood specimen must be adequately mixed before making a blood film or performing a blood count. Mixing for 1 minute on a mechanical rotating mixer is sufficient [37]. Manual inversion (10 times) is also satisfactory provided that any refrigerated samples are first brought to room temperature [37].

Making a blood film

A blood film may be made from non-anticoagulated (native) blood, obtained either from a vein or a capillary, or from EDTA-anticoagulated blood. Chelation of calcium by EDTA hinders platelet aggregation so that platelets are evenly spread and their numbers can be assessed more easily (Fig. 1.6). Films prepared from capillary blood usually show prominent platelet aggregation (Fig. 1.7) and films from native venous blood often show small aggregates (Fig. 1.8). Films prepared from native venous or capillary blood are free of artefacts due to storage or the effects of the anticoagulant. Few laboratories now use such films as a matter of routine, but they can still be useful for investigating abnormalities such as red cell crenation or white cell or platelet aggregation that may be induced by storage or EDTA. Conversely, making a blood film from EDTA-anticoagulated blood after arrival of the blood specimen in the laboratory has the advantage that some of the artefacts that may influence the validity of results obtained from automated instruments are more likely to be detected, for example the formation of fibrin strands, aggregation of platelets or agglutination of red cells induced by cold agglutinins. Good laboratory practice includes recording the date and time the specimen is received in the laboratory and making a film shortly after receipt of the specimen. In this way the length of any delay in transit is known and attribution of morphological changes to prolonged storage of EDTA-anticoagulated blood ('storage artefact', see Chapter 3) can be confirmed.

Blood films are prepared and examined on only a proportion of the specimens on which a blood count is performed. Consensus guidelines are available that suggest appropriate indications for the examination of a film [38]. Clinical details and the results of automated blood counts are both relevant in determining which specimens require a film.

Manual spreading of a blood film on a glass slide (wedge-spread film)

Glass slides must be clean and free of grease. They should not be too porous or background staining is increased [39]. A spreader that is narrower than the slide is required. If a coverslip is to be applied, the spreader must also be narrower than the coverslip so that cells at the edge of the blood film are covered by the coverslip and can be easily examined microscopically. A spreader can be readily prepared by breaking the corner off a glass slide after incising it with a diamond pen; this provides a smooth-edged spreader that is large enough to be manipulated easily. Spreaders made by

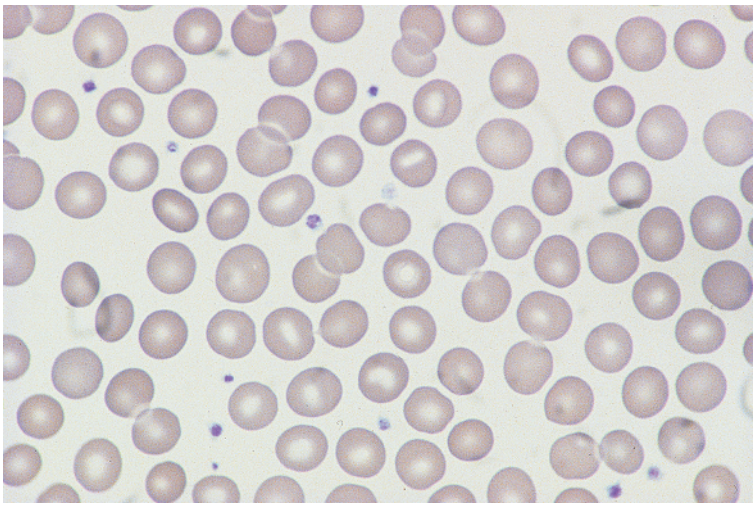

Fig. 1.6 Blood film from ethylenediaminetetra-acetic acid (EDTA)-anticoagulated blood showing an even distribution of platelets.

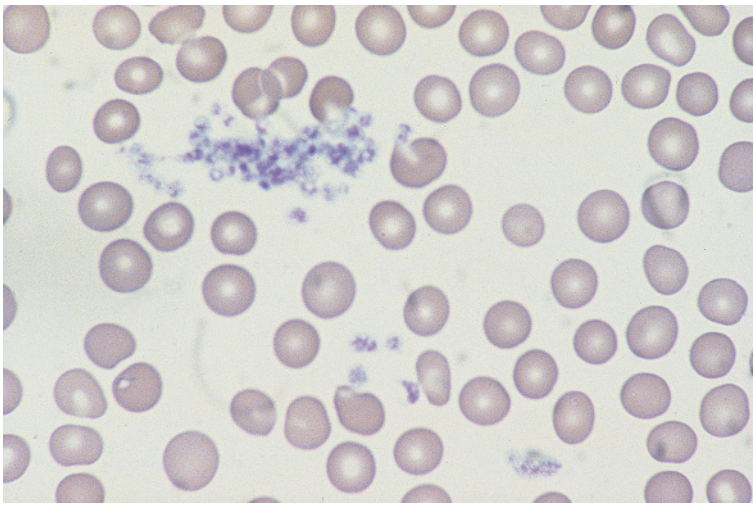

Fig. 1.7 Blood film from non-anticoagulated capillary blood showing the aggregation of platelets that usually occurs.

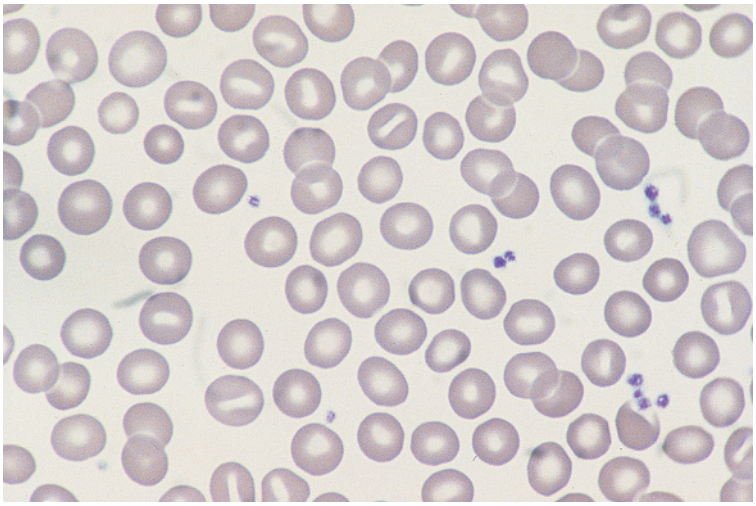

Fig. 1.8 Blood film from non-anticoagulated venous blood showing the minor degree of platelet aggregation that usually occurs.

cutting transverse pieces from a slide are inferior since they are smaller, are more difficult to handle and have at least one rough edge that may damage gloves or fingers.

The laboratory worker spreading blood films should wear gloves. A drop of blood (either native or anticoagulated) is placed near one end of the slide. Anticoagulated blood from a screw-top container can be applied to the slide using a capillary tube, which is then discarded. A drop of blood from a specimen container with a penetrable lid can be applied to the slide by means of a special device that perforates the lid. Once the drop of blood has been placed, the spreader is applied in front of the drop at an angle of 25–30° and is drawn back into it (Fig. 1.9). When the blood has run along its back edge, the spreader is advanced with a smooth, steady motion so that a thin film of blood is spread over the slide. If the angle of the spreader is too obtuse or the speed of spreading is too fast, the film will be too short. An experienced operator learns to recognise blood with a higher than normal Hct, which is more viscous and requires a more acute angle to make a satisfactory film and, conversely, blood with a lower than normal Hct, which requires a more obtuse angle. The spreading technique should produce a film of blood at least 2.5 cm long with a fairly straight tail; it should stop at least 1 cm from the end of the slide. If the film is in the shape of a thumbprint it means that, when observing the film microscopically and moving across the film, the observer

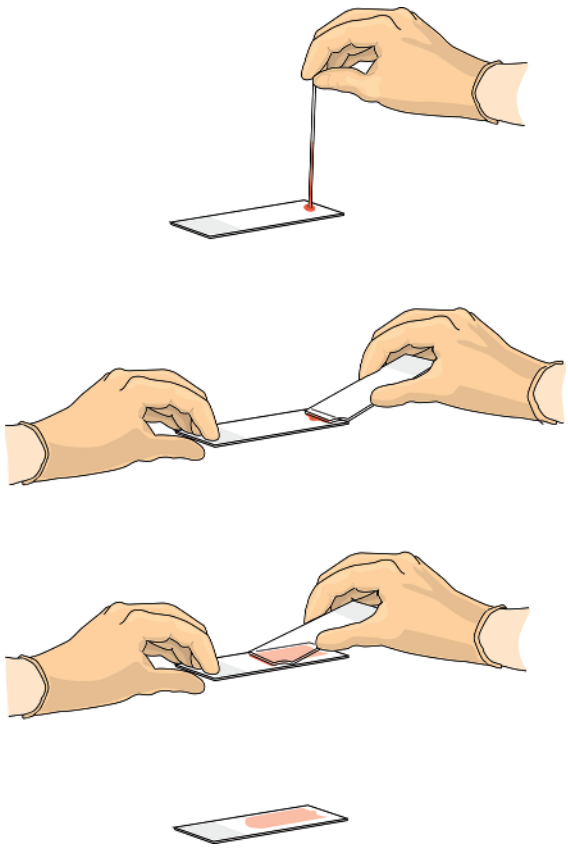

Fig. 1.9 The method of spreading a blood film.

moves from an area that is optimal for identification of cells to an area that is too thick. It is important to wipe the spreader clean with a dry tissue or gauze square after each use to avoid transferring abnormal cells from one blood film to another (Fig. 1.10).

As soon as slides are made they should be labelled with the patient's name and the date or with an identifying number. Small numbers of slides can be labelled with a diamond marker or by writing details on the thick part of the film. The fastest way to label large numbers of slides is with a methanol-resistant pen or by writing in pencil on the frosted end of a slide. Slides that are frosted at one end on **both** sides are useful because they avoid waste of staff time ensuring that the slide is the right way up. Blood films should be dried rapidly. A hot-air blower or a fan to increase air circulation can be useful. If films are dried slowly, there is shrinkage of cells that can lead to the appearance of cytoplasmic blebs and villi, bipolar lymphocytes, hyperchromatic nuclei and inapparent nucleoli [31]; these changes can occur not only in normal cells but also in neoplastic cells so that their characteristic features are less apparent.

Figure 1.11 shows a well-spread film in comparison with examples of poor films resulting from faulty technique.

Unless stated otherwise, this book deals with morphology as observed in wedge-spread films. Most of the photographs are of manually spread films prepared from recently collected EDTA-anticoagulated blood.

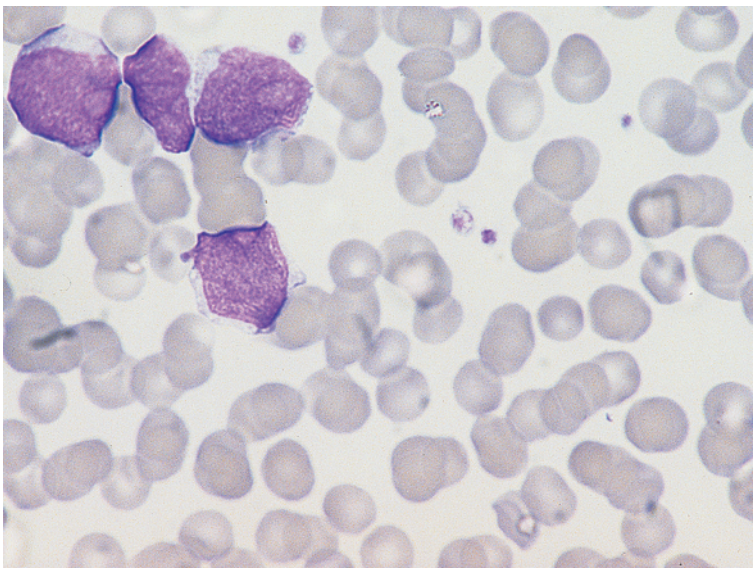

Fig. 1.10 Blast cells from a patient with acute leukaemia that have been inadvertently transferred to the blood film of another patient as a result of using an inadequately cleaned spreader.

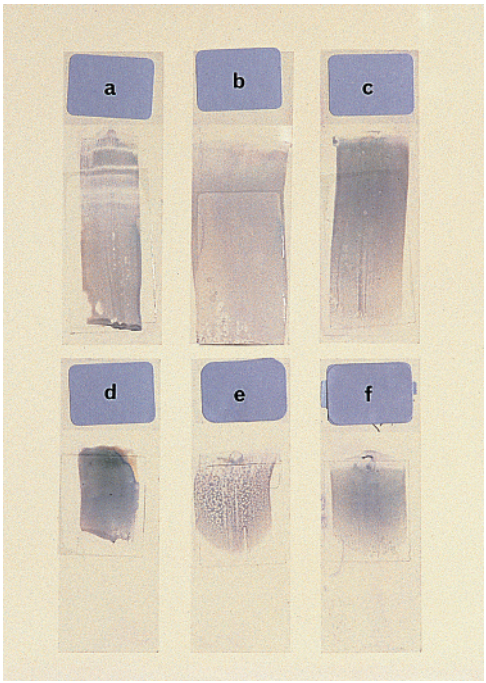

Fig. 1.11 Unsatisfactory and satisfactory blood films: (a) uneven pressure has produced ridges; (b) too broad and too long – the edges and the tail of the film cannot be examined adequately; (c) too long and streaked by an uneven spreader; (d) too thick and short due to the wrong angle or speed of spreading; (e) even distribution of blood cells has been interrupted because the slide was greasy; (f) satisfactory.

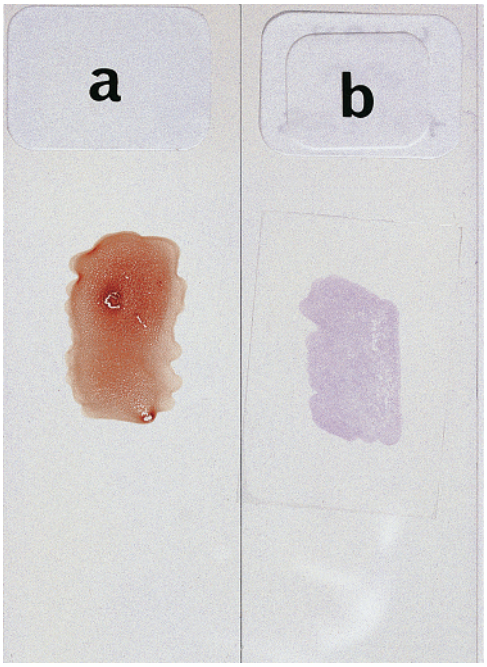

Fig. 1.12 Thick films for examination for malarial parasites: (a) unstained film showing the correct thickness of the film of blood; (b) film stained without fixation, causing lysis of red cells.

Other methods of spreading thin films

Automated spreading of blood films

Wedge-spread films can be prepared by mechanical spreaders, which can be integrated into a staining machine or an automated full blood counter. Such devices can damage cells, altering their cytological features [40]. A film of blood one cell thick can also be spread on a glass slide by centrifugation in a specially designed centrifuge, but this method is little used.

Films from blood with a very high haematocrit

If blood has a very high Hct, for example Hct >0.60, Hb >200 g/l, it can be impossible to make a good blood film, even if the angle and the speed of spreading are adjusted. Mixing a drop of blood and a drop of either saline or blood group AB plasma reduces viscosity so that a film can be made in which details of red cell morphology can be appreciated.

Buffy coat films

Buffy coat films are useful to concentrate nucleated cells, for example to look for low-frequency abnormal cells or bacteria. A tube of anticoagulated blood is centrifuged and a drop of the buffy coat is mixed with a drop of the EDTA-anticoagulated plasma and is spread in the normal manner.

Thick films

Thick films in which the red cells are lysed before the film is examined are required for the detection of malaria parasites and certain other parasites. Parasites are much more concentrated in a thick film, so that searching for them requires less time. To make a thick film, several drops of native or EDTA-anticoagulated blood are placed in the centre of a slide and stirred with a capillary tube or an orange stick to make a pool of blood of such a thickness that typescript or a watch face can be read through it (Fig. 1.12). The blood film is not fixed but, after drying, is placed directly into an aqueous Giemsa stain so that lysis of red cells occurs; this allows the organisms to be seen more clearly.

Unstained wet preparations

Unstained wet preparations are useful for searching for motile parasites such as microfilariae, which can be seen agitating the red cells. A drop of anticoagulated blood is placed on a slide and covered with a coverslip.

Fixation, staining and mounting

Fixation

Following air drying, thin films are fixed in absolute methanol for 10–20 minutes. It is important not to proceed to fixation until the blood film is dry. Premature fixation of a damp film leads to a characteristic artefact in which the contents of the nucleus appear to leak into the cytoplasm (Fig. 1.13). Poor fixation, also with characteristic artefactual changes, occurs if there is more than a few per cent of water in the methanol (Fig. 1.14); this renders interpretation of morphology, particularly red cell morphology, impossible and, if the film is not examined carefully, can give a mistaken impression of hypochromia. In warm, humid climates it may be necessary to change methanol solutions several times a day. Similar artefactual changes can be produced by condensation on slides. In humid climates, slides should be fixed as soon as they are thoroughly dry. A hot-air blower can be used to accelerate drying. In any circumstances, prolonged delay in fixation should be avoided as this can lead to alteration in the staining characteristics of the film, which can acquire a turquoise tint.

Staining

There is little consistency between laboratories as to the precise stain used to prepare a blood film for microscopic examination, but the multiple stains in use are based on the

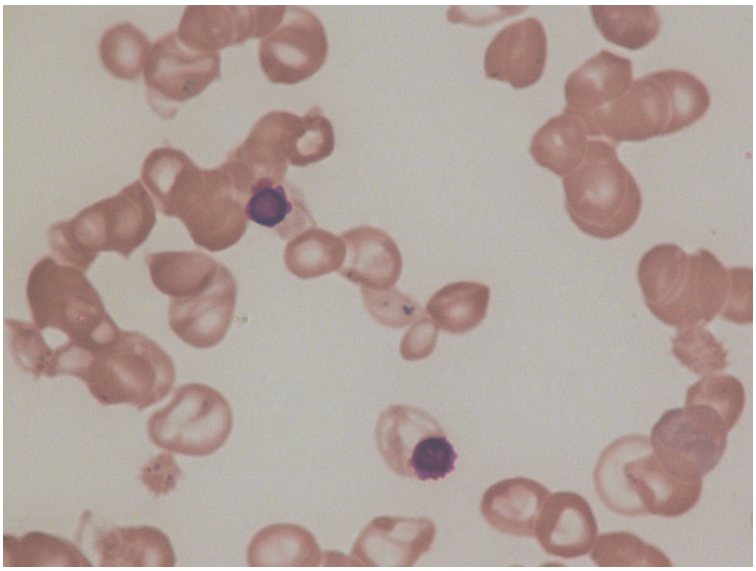

Fig. 1.13 Blood film that has been fixed before drying was complete. It is important not to confuse the apparent leaking of nuclear contents into the cytoplasm, which is an artefactual change, with dyserythropoiesis.

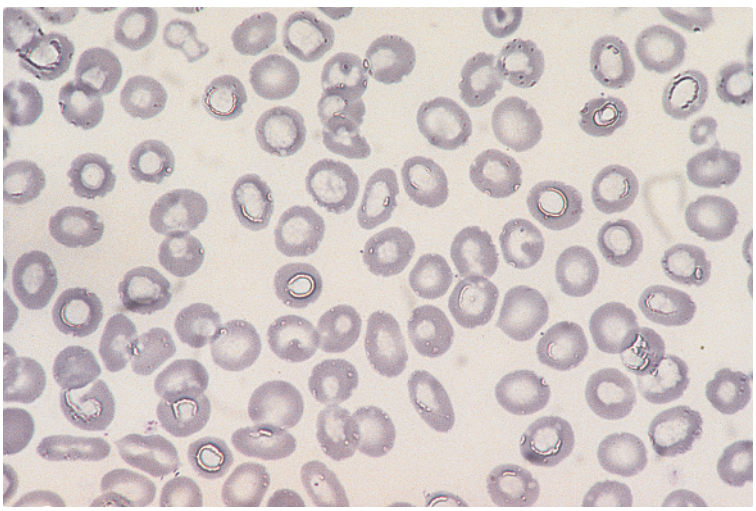

Fig. 1.14 Artefactual changes produced by 5% water in the methanol used for fixation.

Romanowsky stain, developed in the late nineteenth century by Dmitri Romanowsky, a Russian protozoologist [41]. Romanowsky used a mixture of old methylene blue and eosin to stain the nucleus of a malaria parasite purple and the cytoplasm blue. Subsequently, Gustav Giemsa, a German chemist working in Hamburg, modified the stain, combining methylene azure and eosin. The stain most commonly used in the UK is a combination of Giemsa stain with May–Grünwald stain, the latter developed in Munich by a physician called Richard May and an otolaryngologist called Ludwig Grünwald; it is therefore designated the May–Grünwald–Giemsa (MGG) stain. The stain most commonly used in North America is the Wright stain (developed by the Boston pathologist, James Homer Wright in the first decade of the twentieth century), which contains methylene blue and eosin; the methylene blue has been heated, or 'polychromed', to produce analogues of methylene blue. Sometimes this is combined with Giemsa stain to give a Wright–Giemsa stain, which is generally held to give superior results. It has been demonstrated by chromatography that dyes prepared by traditional organic chemistry methods are not pure and that dyes sold under the same designation can contain variable mixtures of five to ten dyes [42]. Variation between different batches prepared by the same manufacturer also occurs.

The essential components of a Romanowsky-type stain are: (i) a basic or cationic dye, such as azure B, which conveys a blue-violet or blue colour to nucleic acids (binding to the phosphate groups of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)) and to nucleoprotein, to the granules of basophils and, weakly, to the granules of neutrophils; and (ii) an acidic or anionic dye, such as eosin, which conveys a red or orange colour to haemoglobin and the eosinophil granules and also binds to cationic nuclear protein, thus contributing to the colour of the stained nucleus. A stain containing azure B and eosin provides a satisfactory Romanowsky stain [41], as does a mixture of azure B, methylene blue and eosin [42]. The ICSH reference method for the Romanowsky stain [43], which uses pure azure B and eosin Y, gives very satisfactory results but such pure dyes are expensive for routine use. Satisfactory and reasonably consistent staining can be achieved using good-quality commercial stains and an automated staining machine. This method has been used for staining the majority of blood films photographed for this book.

Traditionally, cytoplasm that stains blue and granules that stain purple have both been designated 'basophilic', and granules that stain violet or pinkish-purple have been designated 'azurophilic'. In fact all these hues are achieved by the uptake of a single basic dye such as azure B or A. 'Acidophilic' and 'eosinophilic' both refer to uptake of the acidic dye, eosin, although 'acidophilic' has often been used to describe cell components staining pink, and 'eosinophilic' to describe cell components staining orange. The range of colours that a Romanowsky stain should produce is shown in Table 1.2.

Table 1.2 Characteristic staining of different cell components with a Romanowsky stain.

Cell component	Colour
Chromatin (including Howell–Jolly bodies)	Purple
Promyelocyte granules and Auer rods	Purplish-red
Cytoplasm of lymphocytes	Blue
Cytoplasm of monocytes	Blue-grey
Cytoplasm rich in RNA (i.e. 'basophilic cytoplasm')	Deep blue
Döhle bodies	Blue-grey
Specific granules of neutrophils, granules of lymphocytes, granules of platelets	Light purple or pink
Specific granules of basophils	Deep purple
Specific granules of eosinophils	Orange
Red cells	Pink

Staining must be performed at the correct pH. If the pH is too low, basophilic components do not stain well. Leucocytes are then generally pale, with eosinophil granules a brilliant vermilion. If the pH is too high, uptake of the basic dye may be excessive, leading to general overstaining; it then becomes difficult to distinguish between normal and polychromatic red cells, eosinophil granules are deep blue or dark grey, and the granules of normal neutrophils are heavily stained, simulating toxic granulation.

Stain solutions may need to be filtered shortly before use to avoid stain deposit on the blood film, which can be confused with red cell inclusions. If an automated staining machine is used, better results are usually achieved with a dipping technique, in which the entire slide is immersed in the stain, than with a flat-bed stainer, in which staining solution is applied to a horizontal slide. The latter type of staining machine is more likely to leave stain deposits on the slides and, if the blood film is too long or badly positioned, some parts of it may escape staining.

Destaining an MGG-stained blood film can be done by flooding the slide with methanol, washing it in water and then repeating the sequence until all the stain has gone. This can be useful if only a single blood film is available and a further stain, for example an iron stain, is required.

Staining for malaria parasites

The detection and identification of malaria parasites are facilitated if blood films are stained with a Giemsa (or Leishman) stain at pH 7.2. At this pH, cells that have been parasitised by

either *Plasmodium vivax* or *Plasmodium ovale* have different tinctorial qualities from non-parasitised cells and are easily identified. The inclusions in parasitised cells are also evident.

Mounting

If films are to be stored, mounting gives them protection against scratching and gathering of dust. As stated above, the coverslip should be sufficiently wide to cover the edges of the blood film. A neutral mountant that is miscible with xylene is required.

As an alternative to mounting, blood films can be sprayed with a polystyrene or acrylic resin.

If films are not to be stored, a thin film of oil can be smeared on the stained slide to permit microscopic examination at low power before adding a drop of oil to permit examination with the oil immersion lens.

Storage of slides

Ideal patient care and continuing education of haematology staff dictate that blood films should be stored for as long as possible, preferably for some years. Unfortunately, the very large numbers of blood specimens now being processed daily by most haematology laboratories means that this is often difficult. The most economical way to store slides is in metal racks in stacking drawers. Labels showing the patient's name, the date and the laboratory number should be applied in such a way that they can be read when the slides are in storage. Slides that have been freshly mounted should be stored in cardboard trays or stacked in racks, separated from each other by wire loops, until the mountant has hardened and dried. When the mountant is no longer sticky, slides can be stacked closely together for maximum economy of space. Glass slides are heavy and if large numbers are to be stored the floor of the room may need to be strengthened.

When a patient has a bone marrow aspiration performed, a blood film should always be stored permanently with bone marrow films so that when it is necessary to throw out old peripheral blood films to make room for new ones, at least this film is available for review. A laboratory should also maintain a separate file of teaching slides. These should include examples of rare conditions and typical examples of common conditions.

Setting up and using a microscope

All laboratory workers should learn to set up a microscope correctly early in the course of their training. The following is the correct procedure for setting up a binocular microscope.

1. If you need to move or lift the microscope do so using only the arm (Fig. 1.15). Sit at the microscope and make sure that the height is correct for comfortable viewing. Adjust the chair or the height of the microscope above the bench, as necessary.
2. Plug in the electric lead and switch on the mains power supply.
3. Turn on the microscope.
4. Turn up the rheostat until there is a comfortable amount of light.
5. Lower the stage and rotate the ×10 objective into place; it will click when it engages.
6. Select a slide and place it on the stage, being careful to place it with the blood film and coverslip uppermost. Handle the slide only by its edges. Secure the slide with the levers provided for this purpose.
7. Raise the condenser as high as it will go.
8. Open the field diaphragm and the condenser aperture diaphragm fully.
9. Move the stage until the film of blood is beneath the objective, in the beam of light.
10. Raise the stage, looking at the slide from the side or the front rather than using the oculars (eyepieces), until the slide almost touches the objective.
11. Adjust the position of the oculars so that they match your interpupillary distance and look at the slide through the oculars, making sure that the light is at a comfortable level.
12. Lower the slide slowly using the coarse focus knob until the slide comes into focus.
13. Using the coarse and then the fine focus, focus on the slide with your right eye to the right ocular then, without moving the slide, rotate the ring on the left ocular so that the image recorded by your left eye is also sharp. (With some microscopes it is possible to adjust both oculars.)
14. Close the field iris diaphragm fully. The field iris diaphragm is near the lamp and controls the area of illumination.
15. Lower the condenser until the edge of the field iris diaphragm comes into focus. Check that the aperture in the field iris diaphragm is centred and, if it is not, centre it using the two centring knobs on the condenser.
16. Adjust the focus by moving the condenser so that the edges of the diaphragm appear faintly blue rather than faintly red (Köhler illumination).
17. Open the field iris aperture so that the whole field of view is illuminated but no wider. If it is opened too wide, stray light will enter the field of view. This is particularly important for photography, for which purpose the diaphragm can be closed further until only the photographic frame is illuminated.

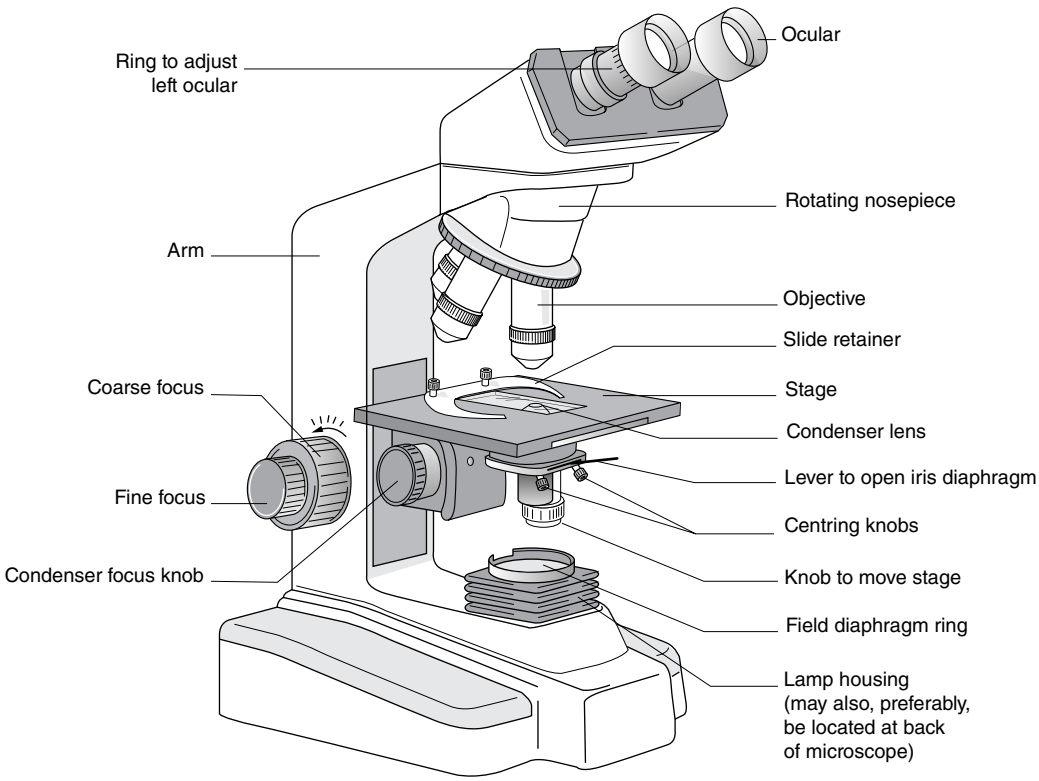

Fig. 1.15 Drawing of a microscope, showing the names of the individual parts.

18 Using the appropriate lever or ring, close the condenser aperture diaphragm to about 70–80% of the numerical aperture marked on the objective. A condenser scale near the lever or ring permits this to be done. This aperture controls the angular aperture of the cone of light that reaches the condenser lens. The more you close this aperture the less light there is and the lower the resolution, but the greater the contrast and depth of focus. For optimal optics, the condenser aperture iris should be reset for each objective.

19 Examine the slide with the ×10 objective*, then rotate in a ×40 objective. Readjust the focus and the condenser aperture iris and adjust the field iris diaphragm so that only the field of view is illuminated. Re-examine the slide.

20 Before using an oil immersion lens, rotate out the non-oil objective and put a drop of immersion oil in the centre of the slide. Rotate in an oil immersion objective, for example ×60 or ×100, and focus, using the coarse and then the fine focus and adjust the condenser aperture iris. Be careful not to rotate in any objective other than an oil immersion objective while there is a drop of oil on the slide. If you are not sure if an objective is for oil immersion or not, read its label. Do not use excess oil and do not mix two different types of oil. Do not overfill a bottle of oil or oil will get on your fingers.

21 After examining a slide with an oil immersion objective, gently wipe the oil from the objective and from the slide, using only a lens tissue on the objective. If the slide has been freshly mounted take care that the coverslip is not accidentally removed; remove enough of the oil to ensure that a non-oil lens will not be contaminated if the slide is again placed on a microscope stage. Removing oil from slides does not require lens tissues; ordinary tissues are satisfactory and cheaper. Non-mounted blood films are not advised but, if they are

*Note: microscopes used by haematology laboratories do not usually have a ×4 objective fitted, unless histological sections are also being examined. If a ×4 objective is to be used, e.g. for examining a trephine biopsy section, swing out the condenser before viewing the slide.

used, be careful to minimise scratching of the blood film when removing oil.

22 When you have finished working, rotate back in the lowest power objective and lower the stage. Remove traces of oil from any oil immersion lens using methanol and lens tissues. Turn down the rheostat before turning off the microscope. Do not leave the microscope turned on when you are away from your workstation; in some poorly designed microscopes the lamp is very close to the field diaphragm and prolonged heat will damage the leaves of the diaphragm.

23 Keep the microscope clean. Dust can be removed with a small brush. Lenses should be cleaned only with lens tissues. These can be moistened with methanol (or a mixture of 3 parts methanol to 7 parts ether).

24 Cover the microscope with a dust cover when not in use.

Identifying the source of a problem and preventing problems

- If there is no light, check that the light beam has not been deflected to a camera.
- If you cannot focus on a blood film, check if the slide is upside down and make sure that there are not two coverslips instead of one. Some microscopes have a 'stop' on the coarse focus; if necessary, release it. Rarely, very thick slides may make it impossible to focus with a high power lens if a coverslip is mounted. Unsuitably thick coverslips can have the same effect.
- If you cannot see the image clearly, clean the slide using tissues and methanol. Sealed methanol-soaked squares of tissue used to prepare the arm for venepuncture are convenient for cleaning slides and avoid the need to have a bottle of methanol in the microscopy laboratory. If cleaning the slide does not help, clean the objective gently using a lens tissue and methanol. Do not use xylene unless you are unable to get the lens clean with methanol.
- If you wear spectacles you will find that it is impossible to use a microscope with bifocal or varifocal lenses. Modern plastic spectacle lenses are easily scratched and if there is an antiglare coating to the lenses this can also be scratched. Make sure that the oculars have a protective rubber guard if you are using spectacles with this type of lens or lens coating.

Examining a blood film

1 Check the label of the slide (patient identity and date).

2 Examine the film macroscopically for unusual characteristics.

3 Adjust the microscope as above and examine the film microscopically, examining the edges and the tail and then the whole film under low power, e.g. ×10 objective.

4 Next examine the whole film with a ×40 or ×50 objective. This is the most important part of the film examination as it is possible to scan the entire film to note any rare abnormal cells. Be systematic: look specifically at red cells, white cells and platelets.

5 Perform a differential count, if indicated.

6 Examine with an oil immersion lens only if there is some particular reason to do so.

7 Remember that all blood films should be examined with a knowledge of the age, gender, ethnic origin and clinical history of the patient, and with an awareness of the results of the FBC and any instrument 'flags' indicating possibly significant abnormalities.

TEST YOUR KNOWLEDGE

Visit the companion website for MCQs and EMQs on this topic:
www.wiley.com/go/bain/bloodcells6e

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