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Blood: tests used to assess the physiological and immunological properties of blood

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Quinn JG, Tansey EA, Johnson CD, Roe SM, Montgomery LEA. Blood: tests used to assess the physiological and immunological properties of blood. *Adv Physiol Educ* 40: 165–000, 2016; doi:10.1152/advan.00079.2015.—The properties of blood and the relative ease of access to which it can be retrieved make it an ideal source to gauge different aspects of homeostasis within an individual, form an accurate diagnosis, and formulate an appropriate treatment regime. Tests used to determine blood parameters such as the erythrocyte sedimentation rate, hemoglobin concentration, hematocrit, bleeding and clotting times, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean cell volume, and determination of blood groups are routinely used clinically, and deviations outside the normal range can indicate a range of conditions such as anemia, pregnancy, dehydration, overhydration, infectious disease, cancer, thyroid disease, and autoimmune conditions, to mention a few. As these tests can be performed relatively inexpensively and do not require high levels of technical expertise, they are ideally suited for use in the teaching laboratory, enabling undergraduate students to link theory to practice. The practicals described here permit students to examine their own blood and that of their peers and compare these with clinically accepted normal ranges. At the end of the practicals, students are required to answer a number of questions about their findings and to link abnormal values to possible pathological conditions by answering a series of questions based on their findings.

blood; erythrocytes; hemoglobin; hematocrit; blood groups

IN THESE PRACTICAL ACTIVITIES, the student's own blood as well as blood that has been typed and obtained from the Northern Ireland Blood Transfusion Service are used to measure a range of physiological and immunological factors in healthy subjects. During the practical, the erythrocyte sedimentation rate (ESR) is determined by measuring the height of the plasma column in a sample of undisturbed blood, hematocrit is determined using a microhematocrit reader, and the hemoglobin content of blood is measured using colorimetric methodology. Students should be able to calculate the following secondary indexes: mean corpuscular/cell volume (MCV), mean corpuscular/cell hemoglobin concentration (MCHC), and mean corpuscular/cell hemoglobin (MCH) from the primary indexes (hematocrit and hemoglobin concentration) and use these calculations to understand the diagnosis of different types of anemias based on these results.

In the latter part of the practical, blood agglutination is observed by mixing an unknown blood type with known blood agglutinins. Based on their observations, students should be able determine the unknown blood type provided and deter-mine their own blood group. Students also measure their coagulation and bleeding times by recording the length of time blood stops moving in a microcapillary tube and the length of time it takes for bleeding to stop after a small prick injury, respectively.

Background

Although the following body of work does not describe an outcome-based educational research experiment, it does highlight the strengths offered by laboratory activities for student learning. The combination of classroom-based theory and the application of these theories in the laboratory are considered as essential components for modern, successful science graduates and future research scientists and have been described as a way to learn with understanding and engage in a process of constructing knowledge by doing science (25).

It has been suggested that the practical skills and conceptualization that laboratory activities offer are essential in providing students experience with equipment, organisms, and chemicals and that they promote important creative and critical generic skills such as report writing, data handling, interpretation skills, and the ability to evaluate evidence (3, 10, 15, 17). Laboratory practicals are not only highly regarded by educators and trainers but also by students themselves, who have commented that practical classes are an invaluable tool for their learning experiences as they illuminate and reinforce theory and provide an opportunity to learn the tools of the trade (30).

Blood samples are routinely used to assess the health of an individual, specify and diagnose different blood-related and non-blood related pathologies, track the progression of disease, and evaluate the efficacy of different treatments. To do this, multiple properties of blood must be investigated and compared with standardized normal values. These parameters include the composition of the blood, including erythrocyte numbers, hematocrit value, hemoglobin concentration, erythrocyte morphology, plasma protein levels, numbers and different classification of leukocytes, numbers of thrombocytes, levels of various common electrolytes and hormones, detection of biochemical markers not normally present in blood, which particular blood group an individual belongs to, and whether or not the hemostatic mechanisms of an individual operates efficiently when required.

It is well established that a major function of erythrocytes is to transport hemoglobin, which, in turn, carries oxygen from the lungs to the tissues to meet their metabolic demands; therefore, any deviation of hemoglobin concentration in the blood outside the defined normal limits has the potential to cause a detrimental effect on oxygen transport. At sea level, the normal average number of red blood cells is 4.7 and 5.2 million $\pm$

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300,000 cells/mm^3 in female and male subjects, respectively, giving average hemoglobin concentrations of 11.5–16 and 13.5–17.5 g/dl, respectively (13). In a standard blood sample, erythrocytes normally contribute between 37% and 47% in female subjects and between 40% and 54% in male subjects (13) to the overall blood sample; this is known as the hematocrit. Deficiencies in dietary nutrients such as iron, folate, or vitamin B$_{12}$ caused by malnutrition, malabsorption, excess blood loss, leukemia, and increased destruction of erythrocytes causes alterations in erythrocyte morphology and numbers and decreased hematocrit values, which, ultimately, adversely affect hemoglobin concentrations and lead to various forms of anemia. On the other hand, an increased hematocrit value caused by an increase in hemoglobin concentration causes polycythemia. Polycythemia can occur as a result of dehydration, when tissues become hypoxic as a result of not enough oxygen in breathed air, i.e., at high altitudes or failure of oxygen delivery to the tissue as observed in cardiac failure, cor pulmoanale or respiratory disease, or due to a genetic abnormality. When polycythemia occurs as a result of hypoxia (known as secondary polycythemia), compensatory mechanisms using erythropoietin can cause the red blood cell count to rise to 6–7 million/mm^3, ~30% above normal (8). Another type of secondary polycythemia called physiological polycythemia is generally observed in populations who live at altitudes of 14,000–17,000 ft, allowing these people to perform reasonably high levels of continuous work in low-oxygen conditions (this physiological polycythemia is exploited by athletes in high-altitude training camps). Although secondary polycythemia increases the viscosity of the blood and blood volume, its effects on the circulatory system are negligible as the decrease in venous return to the heart associated with increased viscosity and the increase in venous return associated with increased blood volume counteract one another and the cardiac output is not far from normal (8). The arterial pressure in people with polycythemia is also normal due to blood pressure-regulating mechanisms; however, these mechanisms can fail, and hypertension can result. On the other hand, a pathological condition known as polycythemia vera (erythremia) can occur as a result of a genetic defect that leads to the overproduction of red blood cells (7–8 million cells/mm^3) and an increase in the hematocrit value to as high as 60–70% (8). In polycythemia vera, the total blood volume can increases to almost twice that of normal, dramatically increasing blood viscosity, which can lead to many blood capillaries becoming plugged (7). Hemoglobin levels can be influenced by the numbers, sizes, and shapes of erythrocytes, which can be examined clinically, and a number of clinical blood tests will specifically target and examine normal erythrocyte morphology, numbers, hemoglobin concentrations, and hematocrit values. These values can then be used to provide secondary information about single red blood cells, which includes the average red blood cell size (MCV), the amount of hemoglobin per red blood cell (MCH), and the amount of hemoglobin relative to the size of the red blood cell (hemoglobin concentration per red blood cell; MCHC). These parameters are all useful in diagnosing and formulating treatments for different types of anemias. Therefore, erythrocyte morphology, numbers, and content are critical factors in their ability to function as they should and deliver oxygen efficiently to body tissues.

There are other characteristics of blood that can also be examined and used to determine the health of an individual. ESR measures the distance that erythrocytes have fallen after 1 h in a vertical column of anticoagulated blood under the influence of gravity and form stacks, known as stacks rouleaux (4, 5, 8). ESR measurement is a widely used, simple, inexpensive, and nonspecific clinical investigation that indirectly measures the degree of inflammation in the body (4, 20, 24). Normal ESR values range from 0 to 20 mm/h in female subjects under the age of 50 yr and 0–30 mm/h in female subjects over 50 yr of age, whereas male values range from 0 to 15 mm/h under 50 yr of age and 0–20 mm/h over 50 yr of age, respectively (5). ESR is increased in physiological conditions including menstruation and pregnancy, after exercise, increased testosterone levels (27), and diseases such as anemia (12), specific cancers (multiple myeloma and lymphoma) (2, 9), kidney disease (20, 21, 29), thyroid disease (1), infections including rheumatic fever and tuberculosis (26, 31), and autoimmune conditions including lupus and rheumatoid arthritis (18). Other pathological conditions, such as congestive heart failure, hypofibrinogenemia, and polycythemia (7, 12, 28), will cause ESR to decrease.

Further important clinical investigations are also used routinely to identify the blood group that an individual belongs to and the efficiency of the patient's hemostatic mechanisms. Four possible erythrocyte phenotypes (groups) have been identified (A, B, AB, and O) and are so named due to the presence or absence of A and B antigens on the cell surface (Fig. 1), body fluids, and tissue/cells surfaces, including epithelial cells, sensory neurons, platelets, and endothelia of blood vessels, and by the presence of natural antibodies against the antigen in blood serum (6, 14). Patients receiving blood transfusions must receive blood from a compatible blood group donor. As well as the ABO blood types, the rhesus (Rh) antigen (usually indicated by the presence of a + or − after the A, B, or O blood group, i.e., A$^+$ indicates the A blood group and the presence of the Rh antigen) is also central when matching blood for transfusion. Therefore, the blood group O$^-$ can be considered the universal donor as it has no A, B, or Rh antigens present to elicit an immunological response by the recipients' antibodies. Conversely, the blood group AB$^+$ is considered the universal recipient as there will be no antibodies present in the plasma of individuals with this blood group. If there is a mixing of incompatible blood groups, an immune reaction takes place, causing red blood cells to agglutinate. It is this immune
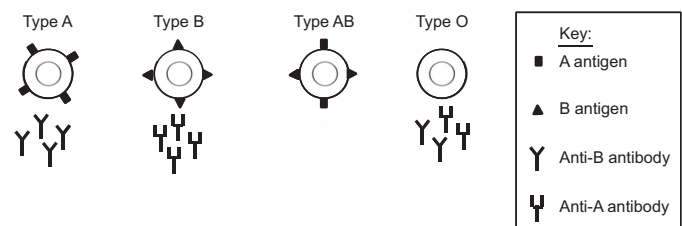

Fig. 1. Diagrammatic representation of the antigens present on each blood type and the antibodies present in the corresponding plasma. Type A blood has erythrocytes with surface A antigens only and plasma containing anti-B antibodies. Type B blood has erythrocytes with surface B antigens only and plasma containing anti-B antibodies. Type AB blood erythrocytes have surface A and B antigens and plasma containing neither anti-A or anti-B antibodies. Type O blood has erythrocytes with neither A or B surface antigens and plasma containing both anti-A and anti-B antibodies.

reaction that forms the basis of blood typing tests often used in student laboratories (22). Aside from agglutination, antibody binding also activates the complement system, resulting in lysis of red blood cells, which leaks hemoglobin into the plasma, which can result in acute renal failure; therefore, it is essential to cross match donor and recipient blood before giving a blood transfusion. The hemostatic system is designed to ensure that there is no major leakage of blood after injury by using a complex system of proteins and enzymes (the coagulation cascade) culminating in the formation of a blood clot. Hemostasis goes through three phases: after damage to a blood vessel, there is an immediate constriction of the damaged vessel, resulting in decreased blood flow and pressure (*phase 1*: vasoconstriction); this is rapidly followed by the formation of a platelet plug, the primary hemostatic plug (*phase 2*). Platelets (thrombocytes) are cell fragments normally numbering between 150,000 and 300,000 platelets/μl (8). When they are activated by exposed collagen from a damaged blood vessel, they undergo a morphological change, developing a spiky outer surface, and release cytokines. These cytokines reinforce vasoconstriction and activate more platelets, which adhere to one another and mechanically block the hole, forming a loose platelet plug; it is the activation of the platelets that begins the clotting process, which ends in the formation of a secondary hemostatic plug in which there is coagulation to prevent blood loss (*phase 3*). The coagulation phase can be divided into two pathways: intrinsic and extrinsic. When damage to the tissue exposes collagen and the intrinsic pathway is initiated, en-

zymes present in the plasma activate factor XII and the coagulation cascade begins. When damaged tissues expose tissue factor (tissue thromboplastin/factor III), factor VII is activated and the extrinsic pathway begins (22). After undergoing a series of reactions, both pathways unite at the common pathway to form fibrin from fibrinogen, the final step of coagulation (Fig. 2). Clinical tests used to measure the time taken for blood to clot and the efficacy of anticoagulant medication are the prothrombin time (PT) and partial prothrombin time (PTT) tests. The PT test evaluates the extrinsic pathway by measuring clotting factors VII, X, and V, prothrombin, and fibrinogen, whereas the PTT test is not dependent on factor VII and therefore assesses the intrinsic pathway by measuring factors XII, XI, IX, VIII, X, and V, prothrombin, and fibrinogen. Normal values for both PT and PTT tests have ranges of 12–16 and 26–37 s, respectively (13). Any disturbances in hemostatic mechanisms can lead to a coagulopathy, resulting in either hypo- or hypercoagulation. Bleeding diathesis states can indicate genetic conditions such as hemophilia or von Willebrand disease, a dietary vitamin K deficiency, or hepatic dysfunctions such as liver disease or failure, resulting in the reduced synthesis of pro- and anticoagulant factors, platelet dysfunction, and quantitative and qualitative thrombocytopenia (19). Plasma or concentrated preparations of factors VIII or IX can be used to treat hemophilia, and vitamin K may be given orally or by injection to promote and or aid coagulation in acquired clotting defects associated with liver disease and vitamin K deficiency. Hypercoagulability caused by thrombotic and
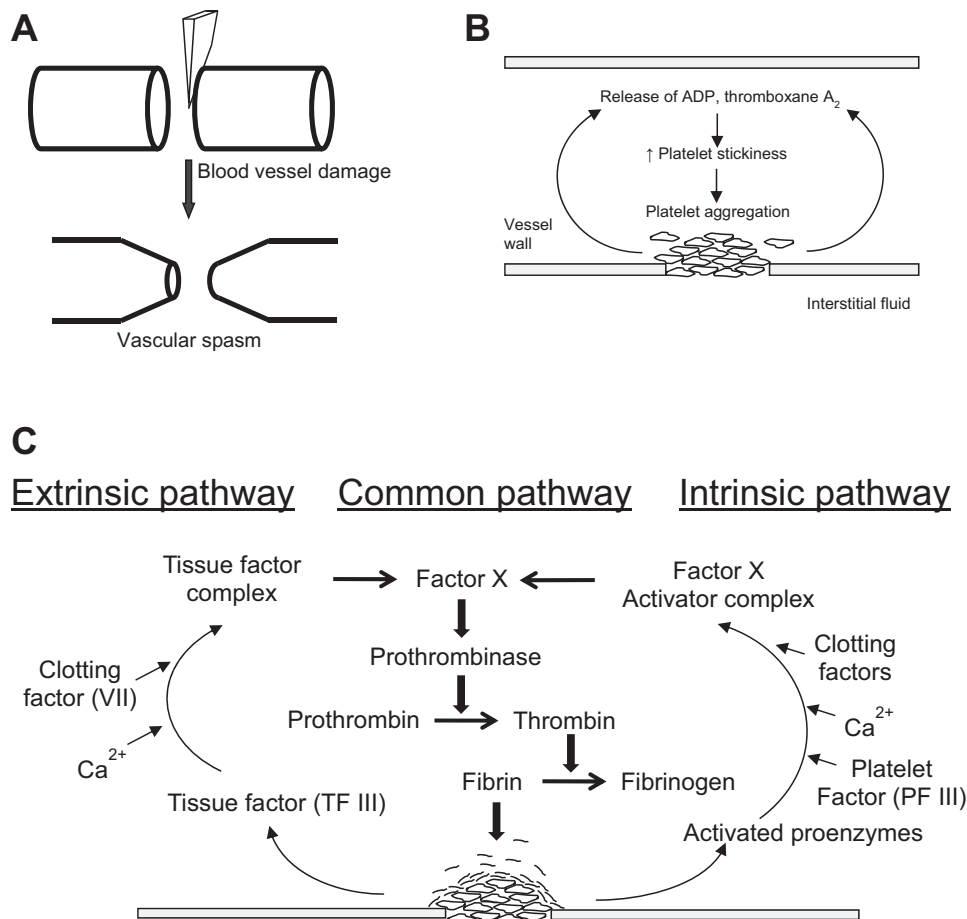
Fig. 2. Diagrammatic representation of the three phases involved in hemostasis. *A*: vascular phase. Endothelial smooth muscle cells contract after injury. *B*: platelet phase. Platelets attach to the endothelial surface, exposed collagen, and each other, promoting chemically mediated platelet activation and further platelet aggregation and vascular spasm. *C*: coagulation phase. This is a series of complex steps, initiated by intrinsic or extrinsic pathways, leading to the formation of factor X and finally culminating in the production of a fibrin network, which traps blood cells and platelets, resulting in the formation of a blood clot.

thromboembolic disease can have severe consequences, including myocardial infarction, stroke, deep vein thrombosis, and pulmonary embolus, and various drugs targeting specific stages of the coagulation cascade are widely used where a coagulopathy is identified or a condition that increases the risk of thrombosis has been identified. Anticoagulants include heparin (which inhibits coagulation by activating antithrombin III), vitamin K antagonists (for example, warfarin), antiplatelet drugs (including aspirin), and fibrinolytic drugs (such as streptokinase).

Learning Objectives

After completing these activities, the student should be able to:

1. Handle blood in a safe and hygienic manner and understand the importance of following these standard operating procedures.

2. Describe the significance of ESR and the clinical possibilities of nonstandard variations and answer the accompanying questions.

3. Account for sex differences in hemoglobin concentrations and the clinical significance of hemoglobin concentrations outside the normal clinical range and answer the accompanying questions.

4. Describe the hematocrit or packed cell volume and recognize the clinical conditions associated with atypical values and answer the accompanying questions.

5. Describe how the hemoglobin concentration and hematocrit values are used to calculate MCV, MCHC, and MCH and how these values are can then be used in the diagnosis and treatment of different anemias and answer the accompanying questions.

6. Perform a slide ABO/Rh blood typing procedure and discuss the clinical significance of ABO/Rh blood typing and how antigens and antibodies relate to the blood typing procedure and answer the accompanying questions.

7. Describe the appearance of the plasma layer and explain why this can sometimes appear cloudy or discolored.

8. Describe the proportion of white blood cells and discuss the factors that may cause this to rise.

9. List the primary, clinically important blood groups of the ABO and Rh systems.

10. Describe the consequences of transfusing incompatible blood.

11. Describe the different phases of hemostasis and how blood loss from the body is prevented by blood vessel constriction and the formation of a platelet plug and the triggering of a blood clot as well as some clinical scenarios that can arise from atypical hemostasis and answer the accompanying questions.

12. Explain the differences between agglutination, cold agglutination, and coagulation.

13. Explain the difference between bleeding and clotting times and the clinical tests used to determine them and what variations in these times can indicate and answer the accompanying questions.

14. Collect and analyze data and draw appropriate conclusions.

Activity Level

These activities are suitable for student studying a variety of courses including human biology, biomedical science, medicine, dentistry, pharmacy, nursing, and the allied health sciences. Currently, these activities are performed by first-year undergraduate medical, dentistry, pharmacy, biomedical science, and human biology students at Queen's University Belfast.

Prerequisite Student Knowledge

Before doing these activities, students should have a basic understanding of:

1. Blood and its components.

2. Hemoglobin and its function.

3. The definition and normal values of the hematocrit, ESR, MCV, MCHC, and MCH.

4. Blood groups and immune responses.

5. Hemostasis and coagulation.

As these experiments use standard laboratory equipment, no specific laboratory skills are required before the practical. Students are introduced to the equipment and shown demonstrations on how to use the equipment at the beginning of the practical class. Teaching staff are close by during the duration of the practical to offer assistance when and where required.

Time Required

The experiments listed below are normally divided into two laboratory-based practical sessions, each lasting 2 h. *Experiments 1–3* are completed in the first 2-h session, and *experiments 4–6* are completed in the second session.

METHODS

Equipment and Supplies

The following equipment and supplies are need for the experiments:

- Gloves
- Safety glasses
- Medi-swabs (cotton wool soaked in 70% alcohol solution)
- Sterile or automated lancets
- Heparinized capillary tubes
- Nonheparinized capillary tubes
- Photoelectric colorimeter
- Cuvettes
- Ferricynaide solution (50 mg/l)
- Syringe (5 ml)
- Pipette (20 μl)
- Pipette tips (20 μl)
- Sharps box
- Capillary tube sealing compound
- Hematocrit tube centrifuge
- Microhematocrit reader
- Unknown blood type
- Student blood sample
- Unknown blood serum
- Anti-A antibody
- Anti-B antibody
- Anti-D (Rh) antibody
- Test tubes
- Pipettes
- Labeled test tube holder (see Fig. 4)
- Glass slides

- Saline
- Blotting paper
- Timer
- Incubator view box

Human Subjects Approval

These experiments do not require ethical approval at Queen's University Belfast. Adopters of this activity are responsible for obtaining permission for human research from their home institution. For a summary of "Guiding Principles for Research Involving Animals and Human Beings," please see www.the-aps.org/mm/Publications/Ethical-Policies/Animal-and-Human-Research.

Safety Considerations

To minimize the risk of infection with blood-borne pathogens, all students should wear laboratory coats, gloves (except when donating a sample of blood), and safety glasses at all times throughout the practical session. Each lancet should be used only once and disposed of in a sharps box designated for disposal for clinical waste immediately after use. Students should handle only their own blood.

The subject should be comfortable with obtaining a small sample of their own blood by a small prick injury administered by a sterile lancet to their finger tip. Subjects should remain seated during the donation of the blood sample.

Individuals with the following conditions should NOT serve as a subject during these experiments:

1. Bleeding disorders such as thrombocytopenia or hemophilia.
2. Individuals with a history of thrombosis, stroke, or liver disease.
3. Individuals taking anticoagulant medication.

Instructions for Experiments

The instructions given to the subjects are as follows.

Experiment 1: Determination of ESR

Step 1. Make sure that the subject's hands are warm.
Step 2. Wipe the skin at the side of the nail of your thumb or finger with a sterile alcohol swab and allow the skin to dry.
Step 3. Uncover the sterile (or automated) lancet and make a frim jab into the prepared skin.
Step 4. Once used, dispose of the lancet into a sharps box immediately.
Step 5. Squeeze the blood toward the pierced skin and try to produce a large drop.
Step 6. Half fill a heparinized capillary tube with the blood obtained by placing the capillary tube into the blood droplet and inverting the tube slightly downward.
Step 7. Seal the tube by pushing it into the sealing compound.
Step 8. Stand the tube vertical and record the time.
Step 9. After an hour, measure the height of the clear plasma zone at the top of the column and record the result (in mm/h; Fig. 3).

Experiment 2: Determination of Hemoglobin Content

Step 1. Transfer exactly 5 ml of ferricyanide diluting solution (50 mg/l) into a clean, dry cuvette using the 5-ml syringe.
Step 2. Obtain a blood sample by repeating *steps 1–5* described in *experiment 1*.
Step 3. Pipette up 20 μl of the blood from the droplet using a fresh pipette tip.
Step 4. Transfer the blood into the ferricyanide solution immediately and mix these by inversion by holding a gloved finger across the top of the cuvette.
Step 5. Leave the sample for 10 min for full color to develop.
Step 6. Blank the colorimeter with distilled water.
Step 7. Place your sample into the colorimeter.
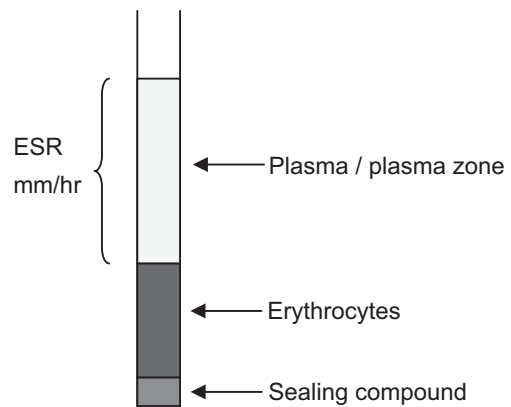*Step 8.* Note the reading.

Fig. 3. Diagrammatic representation of how blood settles in an undisturbed capillary tube and how to measure the erythrocyte sedimentation rate (ESR; in mm/h).

Step 9. Using the chart provided, read off the corresponding concentration of hemoglobin in the blood.
Step 10. Record your result.

Experiment 3: Determination of Hematocrit

Step 1. Obtain another drop of blood by repeating *steps 1–5* described in *experiment 1*.
Step 2. Fill the heparinized capillary with blood to ~1 cm from the top.
Step 3. Seal the tube by pushing it into the sealing compound.
Step 4. Transfer the sealed capillary tube to a centrifuge and spin it at 12,000 *g* for 5 min.
Step 5. Carefully transfer the spun capillary tube to a microhematocrit reader.
Step 6. Record your result.

Experiment 4: Identifying Unknown Blood Types and ABO Grouping by the Tube Technique

Students are supplied with sera and washed blood cells (blood cells that have already been typed and separated). The tubes containing these are placed in a white holder and labeled as shown in Fig. 3. *Tubes 1* and *5* already contain erythrocytes of an unknown blood group, *tubes 2* and *6* contain erythrocytes belonging to blood group A, *tubes 3* and *7* contain erythrocytes belonging to blood group B, and *tubes 4* and *8* contain erythrocytes belonging to blood group O. Students must pipette erythrocytes belonging to blood group A and the unknown blood serum as well as erythrocytes belonging to blood group B and the unknown blood serum into *tubes 9* and *10*, respectively (Fig. 4).

Step 1. Lightly shake the stock tubes to resuspend the cells before discharging the pipette.
Step 2. Using the pipette, add one drop of the appropriately typed cells as indicated on the rack (i.e., pipette A cells into *tubes 2* and *6*; see Fig. 4) to each tube (*tubes 1–10*). Rinse the pipette in the saline solution provided ~10 times between solutions until there are no visible traces of the blood cells to thoroughly wash the pipette and avoid cross-contaminating the reagents. Take care as not to add any reagents or blood to an unlabeled tube.
Step 3. Gently shake the tubes to mix the contents. Allow the tubes to stand at room temperature (~20°C) for 10 min or until the cells visibly clump together (it may occasionally take up to 30 min).
Step 4. Remove the tubes, one at a time, shake them to suspend the red blood cells, and look for clumps of cells that are not dispersed by the agitation. This indicates that agglutination has occurred.
Step 5. Record whether agglutination or not has occurred in each of the tubes.
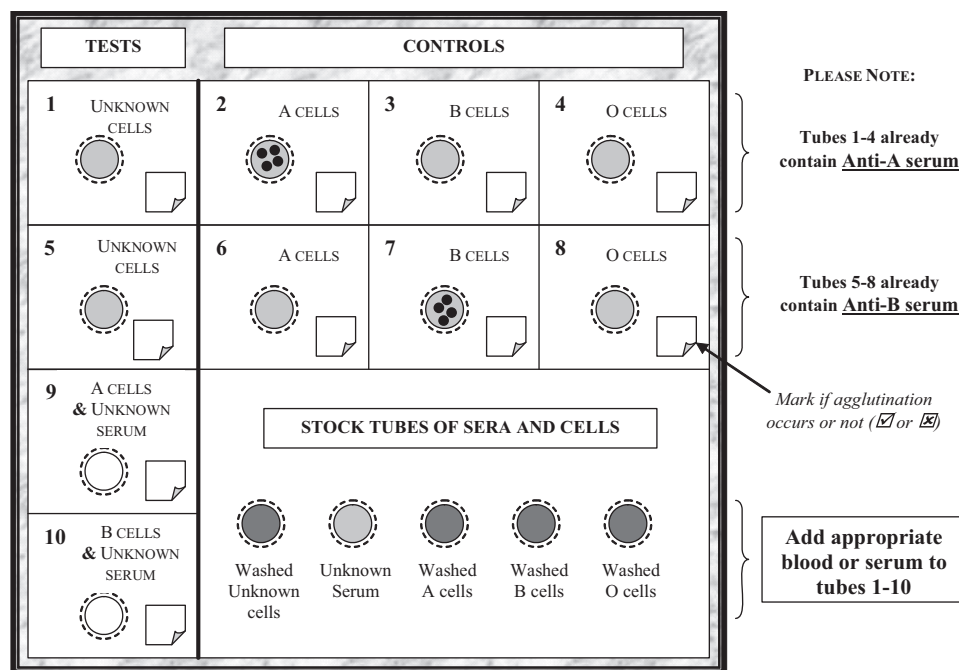
Fig. 4. Rack containing and typed blood sera and cells. Dots represent agglutination in the tubes where it should occur.

Experiment 5: Identifying Unknown Student Blood types and ABO Grouping by the Slide Technique

Step 1. Place one drop each of the anti-A serum, anti-B serum, and isotonic saline onto the appropriate section of a glass slide, divided into three equal sections (Fig. 5).

Step 2. Prick a finger by wiping the skin at the side of the nail of your thumb or finger with a sterile Medi-swab and allow the skin to dry.

Step 3. Uncover the sterile lancet and make a frim jab into the prepared skin.

Step 4. Add as much blood as possible without exceeding four drops into 0.5 ml of isotonic saline in the small test tube.

Step 5. Quickly mix the sample by inverting the tube (using a gloved finger) to leave a pink suspension of red blood cells.

Step 6. Add one drop of red cell suspension to each drop of reagent on the slide using the pipette. Gently shake the slide from side to side to mix or change the pipette tip and stir the cells and reagent on the slide.

Step 7. Wash the pipette in fresh saline between mixing to avoid cross-contamination.

Step 8. When everything has been pipetted out, continue to mix by carefully rocking the slide from side to side for a few minutes.

Step 9. There should be NO agglutination of the blood when it is mixed with saline. From your observations, determine your own blood group. Note if there is agglutination (Fig. 6) of the blood sample when it is mixed with anti-A serum, with anti-B serum, with both, or with none at all. If agglutination is present with anti-A serum ONLY, the blood belongs to blood group A; if agglutination is present with anti-B serum ONLY, the blood belongs to blood group B; if agglutination is present with both anti-A serum and anti-B serum, the blood belongs to blood group AB; and if there is no agglutination when blood is mixed with anti-A serum or anti-B sera, the blood belongs to blood group O.

Fig. 5. Diagrammatic representation of a glass slide divided into three sections with anti-A and anti-B sera and isotonic saline.

Experiment 6: Rh Grouping

Step 1. Repeat *steps 2* and *3* described in *experiment 5* and apply one drop of your undiluted blood to the middle of a plain glass slide.

Step 2. Place one drop of anti-D blood typing serum beside it.

Step 3. Mix the two drops on the slide as described above.

Step 4. Place the slide on top of the preheated (37°C) incubator view box.

Step 5. Tilt the slide backward and forward.

Step 6. If the antigen is present, agglutination will start within a few seconds and be completed within 2 min.

Experiment 7: Coagulation of blood

Step 1. Repeat *steps 1–5* described in *experiment 1*.

Step 2. Half fill a glass capillary tube with the blood obtained by placing the capillary tube into the blood droplet and inverting the tube slightly downward.

Step 3. Tilt the tube so that the column of blood flows ~1 cm toward the other end and then continue to run the column backward and forward at ~15-s intervals.

Step 4. Record the time taken for the blood to become too viscous to move and record this as the clotting time.

Fig. 6. Image showing rhesus D positive red blood cell agglutination when exposed to anti-rhesus D antibodies.

Experiment 8: Bleeding

Step 1. Swab an area of skin on an ear lobe with a sterile Medi-swab and allow the skin to dry.

Step 2. Puncture the skin with a sterile lancet and note the time.

Step 3. Gently blot away all the blood with sterile blotting paper.

Step 4. Repeat the blotting every 30 s until there is no more bleeding and record this time as the bleeding time.

Troubleshooting

Experiments 1–3 and 5–7. To maximize the collection of blood when obtaining a blood sample, keep hands below the level of the heart and ensure that the hands are warm by running them under hot water.

Experiment 4. Both positive control tubes (*tubes 2* and *7*) should show agglutination. *Tubes 3*, *4*, *6*, and *8* should show no agglutination; however, it may appear that agglutination has occurred as red blood cells form rouleaux if left undistirbed or will appear to cluster together at temperatures below body temperature. If this occurs, the tubes should be hand warmed and gently shaken and the cells ought to redisperse. If the cells do not redisperse in any *tubes 3*, *4*, *6*, or *8*, this indicates that an experimental error has occurred, most likely the result of cross-contamination of different types of red blood cells due to inappropriate rinsing of the pipettes (*experiment 4*, *step 2*).

RESULTS AND DISCUSSION

Expected Results

Expected results of the experiments are shown in Tables 1 and 2.

Evaluation of Student Work

Students should record their ESR and hematocrit values and hemoglobin. These results should then be used to calculate MCV, MCHC, and MCH values.

Questions

Question 1. What is the normal ESR range? Does your ESR value lie within the normal range? What could high and low ESR values indicate?

ANSWER. In female subjects, normal ESR is ~0–20 mm/h under 20 yr of age and 0–30 mm/h at 50 yr of age and over. In male subjects, the normal ESR for male subjects under 20 yr of age is 0–15 mm/h and increases to 0–20 mm/h over the age of

Table 1. *Percentage of the population in the United Kingdom, Ireland, and first-year medical students at QUB from 2010 to 2015 expressing the blood antigens A, B, and O*

Blood Group	Percentage of the Population in United Kindom	Percentage of the population in the Republic of Ireland	Percentage of Medical Students at QUB
O^+	44	47	36.7
A^+	28	26	21
B^+	8	9	9.6
AB^+	2	2	5.4
O^-	9	8	15
A^-	6	5	7.1
B^-	2	2	3.8
AB^-	1	1	1.3

$n = 705$ students from Queen's University Belfast (QUB). +/− indicates the presence of rhesus factor D. Values were obtained from the Northern Ireland Transfusion Services, Irish Blood Transfusion Services, and QUB (11, 16).

Table 2. *Normal values for blood indexes and bleeding and clotting times*

	Normal Values
Erythrocyte sedimentation rate, mm/h	
Female subjects < 20 yr old	0–20
Female subjects > 50 yr old	0–30
Male subjects < 20 yr old	0–15
Male subjects >50 yr old	0–20
Hemoglobin concentration, g/dl	
Female subjects	11.5–16
Male subjects	13.5–17.5
Hematocrit value, %	
Female subjects	37–47
Male subjects	40–54
Mean corpuscular/cell volume, fl	80–96
Mean corpuscular/cell hemoglobin concentration, g/100 ml	32–36
Mean corpuscular/cell hemoglobin, pg	27–32
Bleeding time, min	1–6
Clotting time, min	6–10

Values are from Refs. 4, 5, 8, 12, and 13.

50 yr (4, 5). ESR values might be higher than that of the normal range in physiological conditions such as pregnancy, menstruation, and after exercise and pathophysiological conditions including anemias, liver and connective tissue disease, and infections, as high ESR values are associated with an increase in immunoglobulins.

Question 2. Why is a heparinized tube used in this experiment?

ANSWER. Heparin acts as an anticoagulant and prevents the formation of blood clots.

Question 3. What are the normal hemoglobin concentrations for each sex? Does your hemoglobin concentration lie within the normal range for the appropriate sex? What could high and low hemoglobin concentrations indicate?

ANSWER. Normal hemoglobin concentrations range between ~11.5–16 and 13.5–17.5 g/dl for female and male subjects, respectively (13). The hemoglobin concentration in blood can be decreased if the person is suffering from anemia due to a dietary deficiency or excess blood loss or during the female menstrual cycle, whereas it will be increased if the person has been recently living at higher altitudes for a sustained period of time or suffering from polycythemia.

Question 4. What are the normal hematocrit values for each sex and what are possible causes of sex differences in hematocrit values? Does your hematocrit value lie within the normal range for the appropriate sex? What could high or low hematocrit values indicate?

ANSWER. Approximate normal hematocrit values for female and male subjects are between 37–47% and 40–54%, respectively. Sex differences can be accounted for by testosterone levels, muscle mass, and menstruation. Elevated hematocrit values are observed in patients suffering from hypoxia, cor pulmonale, polycythemia, pulmonary fibrosis, congenital heart disease, dehydration, and eryhrocytosis, whereas decreased hematocrit values can be seen in patients with anemia, excessive bleeding, overhydration, malnutrition, leukemia, and increased destruction of red blood cells.

Question 5. What is the color of the plasma layer? Account for possible differences in the color of the plasma layer.

ANSWER. The plasma layer is clear/yellow. If it is pink or colored slightly pink, it suggests a degree of hemolysis.

Question 6. Why is plasma sometimes clear and sometimes cloudy?

ANSWER. This is dependent on the lipid content of the blood. The higher the lipid concentration, the cloudier or more yellow the plasma will appear.

Question 7. What do you roughly estimate to be the proportion of white blood cells? Why might the proportion of white blood cells rise?

ANSWER. The proportion of white blood cells in an overall blood sample usually accounts for <1%. However, the white blood cell count may rise in response to a pathogenic threat from bacteria or parasitic infection, increased inflammation, an allergic reaction, and in conditions that have resulted in tissue death, such as a heart attack or burns. The white blood cell count may also be raised in some cancers, such as myeloproliferative neoplasms and different types of leukemia.

Question 8. From the equations below and following the worked examples, calculate the secondary indexes (average normal value ranges are provided; see Table 2) from the results obtained above.

$$MCV : Hct/100 \times 1/5 \times 10^{12} = \underline{\hspace{2cm}} fl$$

$$MCH : Hb/5 \times 10^{12} = \underline{\hspace{2cm}} pg$$

$$MCHC : Hb \times 100/Hct = \underline{\hspace{2cm}} g/100\ ml$$

where Hct is hematocrit and Hb is hemoglobin.

WORKED EXAMPLES. A female medical student has a hematocrit of 45% and hemoglobin concentration of 15 g/dl. Assume that the red blood cell count per liter of blood is 5×10^{12}. Using these results, what are her MCV, MCH, and MCHC?

- MCV: MCV is the average red blood cell size (8) and is expressed in femtoliters (10^{-15} liters).

MCV can be calculated as follows: MCV = Hct/red blood cell count; Hct/100 × 1/5 × 10^{12}, i.e., 0.45/(5×10^{12} cells) = 0.090×10^{-12} l/cell = 90 fl.

- MCH: MCH is the amount of hemoglobin per red blood cell (8) and is expressed in picograms.

MCH can be calculated as follows: MCH = Hb/5 × 10^{12}. With a hemoglobin of 15 g/dl and a cell count of 5×10^{12} cells per liter of blood, each liter of blood will contain 150 g of hemoglobin.

A single cell will contain 150/(5×10^{12}) = 150/5 pg = 30 pg.

- MCHC: MCHC is the amount of hemoglobin relative to the size of the red blood cell (hemoglobin concentration per red blood cell) (8).

MCHC can be calculated as follows: MCHC = Hb × 100/Hct, i.e., 15 × 100/45 = 15 × 2.22 = 33.3 pg.

Question 9. How would you determine the type of anemia based on MCV and MCHC laboratory values?

ANSWER. The MCV of red blood cells can be used to classify three major types of anemia. If microcytes (smaller than normal erythrocytes) are present, MCV will be low (<80 fl) along with a low MCHC value (<32–36 g/dl). These results indicate hypochromic microcytic anemia. If larger than normal erythrocytes (macrocytes) are observed, MCV can be high (>96 fl) and MCHC is normal; macrocytic anemia can be diagnosed. However, an elevated MCV can be reported with elevated MCHC, suggesting macrocytic hyperchromic anemia. If laboratory values report a normal MCV and normal-sized erythrocytes, normochromic normocytic anemia is diagnosed. However, as MCHC is a measure of hemoglobin concentration, its value is normal with macrocytic anemia. High MCHC values are recorded when sphere-shaped erythrocytes are produced, such as those observed in hereditary spherocytosis and sickle cell disease.

Question 10. What is the likely cause of microcytic anemia?

ANSWER. Iron deficiency is a major cause of microcytic anemia. Iron is an essential component of the heme molecule that combines with globin to form hemoglobin. An inadequate supply of iron to the body due to increased demands such as growth or pregnancy, a deficiency of dietary iron, or malabsorption of iron reduces the formation of hemoglobin. Therefore, erythrocytes contain less hemoglobin than normal, and, as a result, they are hypochromic and microcytic. Other causes of microcytic anemia in which there is a defect in globin synthesis are anemia of chronic disease sideroblastic anemia and thalassemia (13).

Question 11. What is the likely cause of macrocytic anemia? What is the difference between macrocytic and megaloblastic anemia?

ANSWER. Macrocytic anemia can be divided into megaloblastic and nonmegaloblastic types. Vitamin B_{12} and folate (folic acid) are essential for the synthesis of DNA, replication of genes, and production of new cells. They are particularly important at sites of rapid turnover, such as the bone marrow. A deficiency in either one or both of these vitamins by either a dietary deficiency or, in the case of vitamin B_{12} malabsorption, due to the inability of gastric mucosa to produce intrinsic factor (causing pernicious anemia) will adversely affect erythrocyte production. In these circumstances, erythroblastic cells (immature erythrocytes) of the bone marrow will not proliferate fast enough and will also differentiate into macrocytes, larger than normal erythrocytes with a large oval shape (rather than being biconcave) and a weaker cell membrane. Abnormal erythrocyte precursors, megaloblasts, will also be found in the blood. Ultimately, fewer red blood cells are produced, although they are still able to carry oxygen as MCH may be increased slightly, but their fragility causes them to have a shorter lifespan in the circulation. Megaloblastic anemia not only affects erythrocytes but all cell lines and leads to hypersegmented neurophils and pancytopenia. It can also be caused by congenital enzyme deficiencies in DNA synthesis, e.g., orotic aciduria, and drugs that interfere with DNA synthesis (such as hydroxycarbamide), or myelodysplasia due to dyserythropoiesis (13).

Macrocytic anemia without megaloblasts has only large erythrocytes and does not affect all cell lines. It is associated with pregnancy, and common pathological causes include excessive alcohol consumption, liver disease, reticulocytosis, hypothyroidism, and drugs such as cytotoxics (13).

Question 12. What is the likely cause of normocytic anemia?

ANSWER. Normocytic anemia can be caused decreased concentrations of erythropoietin associated with chronic kidney disease or failure of the bone marrow to produce sufficient erythrocytes (aplastic anemia). Aplastic anemia can result from

Table 3. *Blank table for question 18*

Blood Group	Can Donate To	Can Receive From
O^+		
A^+		
B^+		
AB^+		
O^-		
A^-		
B^-		
AB^-		

viral infections, radiation or chemotherapy, autoimmune disorders, drugs, or exposure to certain toxins.

Excessive blood loss (hemorrhagic anemia) causes erythrocytes to leave the circulatory system quicker than they can be replaced and can also cause normocytic anemia; however, chronic blood loss gives rises to microcytic, hypochromic anemia. Another cause may be hemolytic anemia, which is generally a hereditarily acquired condition causing excessive destruction of erythrocytes. The erythrocytes that are produced with this condition are much more fragile than normal and rupture easily as they pass through capillaries, especially those of the spleen, producing schistocytes (fragmented parts of red blood cells). Thus, the lifespan of the fragile cell is so short that the cells are destroyed faster than they are produced.

Normocytic anemia can also be observed with uncompensated increases in plasma volume associated with pregnancy and fluid overload.

Question 13. In some types of anemias, there is a high proportion of reticulocytes. What is the reason for this and the likely underlying pathology?

ANSWER. The reticulocyte count will increase when there is an increased need for erythrocyte production due to blood loss or hemolytic anemia. The number of reticulocytes in the blood will also be increased due to hypoxia and poor renal blood flow due to the effects of erythropoietin and similarly, in the short term, when a person is responding to treatment for anemia or has recently donated blood. This indicates that that the bone marrow is responding to the effects of erythropoietin, stimulating erythrocyte production, due to treatment or clinical intervention or the increased need for erythrocyte production after donation. Although there is an increased need for erythrocyte production in microcytic (iron deficiency) and macrocytic anemia (vitamin B_{12} or folic acid deficiency), the reticulocyte count will actually decrease. With macrocytic anemia, there will be an increase in the number of abnormal, larger erythrocyte precursors (megaloblasts).

Question 14. Using the tube technique, based on whether agglutination occurred when the unknown cells were added to *tubes 1* and *5* (containing anti-A and anti-B sera, respectively), identify what blood group to which the unknown cells belonged. Also using the tube technique, based on whether agglutination occurred when the unknown serum was added to *tubes 9* and *10* (containing A and B cells, respectively), identify the unknown serum.

Question 15. How would you spot a false positive due to cold agglutination?

ANSWER. If there appears to be agglutination in the saline/control droplet (cold agglutinin), it will be dispersed by heating to room temperature or gentle agitation.

Question 16. Using the slide technique, what is your ABO group? Are you D negative or D positive? How did you arrive at these conclusions?

ANSWER. In a positive test, red blood cells exhibiting the Rh antigen will agglutinate in the presence of the anti-D antibody (Fig. 6). This reaction can be visualized on the slide as the red blood cells clump together and form solid masses that will not be dispersed with gentle agitation of the slide. There will be no agglutination in the absence of the Rh D antigen.

Question 17. Explain why someone who is D negative cannot be described as Rh negative.

ANSWER. Other Rh antigens may be present; there are six common types of Rh antigens: C, D, E, c, d, and e. To be truly Rh negative, you must not express C, D, or E antigens; Rh D is the most common and clinically a person who has this antigen is considered to be Rh positive, whereas a person that doesn't express the Rh D antigen is considered Rh negative.

Question 18. Complete the table (Table 3) by determining the ABO blood type the donor must have to give blood to each of the following recipients.

ANSWER. The correct answers are shown in Table 4.

Question 19. What transfusion reactions would occur if there is a mixing of incompatible blood groups?

ANSWER. Transfusion reactions can either be immunological or nonimmunological. Immunological reactions include hemolytic transfusion reactions due to ABO incompatibly. Antibodies in the recipient's blood will attack the antigens present on the donor's erythrocytes, causing the red blood cells to agglutinate (clump together), which may then cut off or reduce the blood supply to various parts of the body. Due to activation of the complement system, hemolysis can occur and hemoglobin will leak into the bloodstream, which can result in acute renal failure. There may be alloimmunization to many less common antigens on erythrocytes and antigens present on leucocytes, platelets, plasma proteins, and during pregnancy to fetal antigens inherited from the father and not shared by the mother. Alloimmunization may not cause clinical problems after the first transfusion upon first exposure to the antigen, but problems may arise with subsequent transfusions (13). Nonhemolytic (febrile) immune-mediated transfusion reactions are common in patients who have previously been transfused or pregnant due to the presence of leucocyte antibodies in an alloimmunized recipient against donor leucocytes in erythrocyte concentrates or the release of cytokines from donor leucocytes in platelet concentrates (13).

Nonimmune transfusion reactions can also occur; these include the transmission of blood-borne viral, bacterial, parasitic, or prion infections such as human immunodeficiency virus (HIV), syphilis, malaria, and Creutzfeldt-Jakob disease, re-

Table 4. *Completed table for question 18*

Blood Group	Can Donate To	Can Receive From
O^+	O^+, A^+, B^+, AB^+	O^+, O^-
A^+	A^+, AB^{+-}	A^+, A^-, O^+, O^-
B^+	B^+, AB^+	B^+, B^-, O^+, O^-
AB^+	AB^+	All blood groups
O^-	All blood groups	O^-
A^-	A^-, A^+, AB^-, AB^+	A^-, O^-
B^-	B^-, B^+, AB^-, AB^+	B^-, O^-
AB^-	AB^-, AB^+	AB^-, A^-, B^-, O^-

spectively. Multiple transfusions can lead to iron overload, and massive transfusion may cause bleeding reactions or electrolyte imbalances (13).

Question 20. What is hemostasis? Briefly discuss its phases.

ANSWER. Hemostasis is the term that summarizes the mechanisms that keeps blood in a fluid state and those which prevent blood loss. There are four phases of hemostasis.

Phase 1: vascular constriction. When a blood vessel has been cut or ruptured, the smooth muscle of the vessel wall will contract, reducing the flow of blood from the injured vessel (Fig. 2*A*).

Phase 2: formation of a platelet plug. The cut in the vessel will be temporarily sealed by platelets. The damaged endothelium of the blood vessel will release von Willebrand factor, which enhances platelet adhesion to the endothelium by forming a bridge between the platelets and exposed collagen. This allows platelets to accumulate at and adhere to the site of injury. Once adhered, platelets will swell and secrete ADP and thromboxane A_2, which, in turn, will attract more platelets to the site of injury and adhere, thus forming a primary platelet plug (Fig. 2*B*).

Phase 3: formation of a blood clot. Activator substances from the cut vessel wall, and platelets and blood proteins adhering to the vessel wall will initiate the clotting cascade. At the end of this process, the hole in the vessel is filled by a clot comprising a meshwork of fibrin fibers, which entraps blood cells, platelets, and plasma (Fig. 2*C*).

Phase 4: clot retraction. The clot retracts and expels serum. As the clot retracts, the edges of the damaged vessel are pulled together.

Question 21. What are your bleeding and clotting times? What are the average bleeding and clotting times? What could prolonged PT and PTT test times indicate?

ANSWER. Average bleeding time is 1–6 min depending on the depth of the wound and degree of hyperemia in the ear at the time of the test (8). Prolonged bleeding times are found in people with the platelet disorder thrombocytopenia. The observed thrombocytopenia may have two causes: impaired platelet production due to congenital defects, cytotoxic drugs, or chemicals, viruses (i.e., HIV), or leukemia or be caused by excessive destruction of the platelets by autoimmune conditions or drugs.

Average clotting time using the glass capillary tube method is 6–10 min (8). Substances used in the clotting process are formed in the liver and require vitamin K. Therefore, acute or chronic liver disease can affect an individual's ability to synthesize the necessary coagulation factors needed for the coagulation phase of hemostasis.

More specific coagulation tests include PT and PTT tests. The PT test measures the coagulation factors involved in the extrinsic coagulation pathway, such as factors VII, X, and V, prothrombin, and fibrinogen, and normally ranges between 12 and 16 s. Prolonged times are associated with liver disease and warfarin therapy (13). The PTT test (normal time: 26–37 s) evaluates the intrinsic coagulation pathway by measuring coagulation factors (factors XII, XI, IX, VIII, X, and V, prothrombin, and fibrinogen). Test times are prolonged when there are deficiencies in one or more of the relevant factors (12).

Question 22. What other test could be used to assess platelet function?

ANSWER. Platelet function may be measured by the platelet function assay.

Question 23. Explain why bleeding and clotting times are different.

ANSWER. Bleeding time measures the length of the vascular and platelet phases the first two phases of hemostasis, which are fast-acting physical processes. Clotting time measures the third phase of hemostasis alone, which takes longer (coagulation cascade). In some individuals, the clotting time may be longer.

Question 24. Compare a platelet plug with a clot.

ANSWER. A platelet plug has a mass of large numbers of platelets with no fibrin (temporary structure), and a clot has fibrin, which reinforces the plug (more permanent structure).

Question 25. What are the differences between the intrinsic and extrinsic coagulation pathways?

ANSWER. When damage to the tissue exposes collagen to the blood, the intrinsic pathway is initiated; enzymes present in the plasma activate factor XII and platelets, which release platelet phospholipids that contain platelet factor 3. Factor XII is activated and then factor XI is activated, which, in turn, activates factor IX. Factor X is then activated by factor IX, factor VIII, and platelet phospholipids (factor III). The extrinsic pathway begins blood contacting a traumatized vascular wall or extravascular tissue. This causes the release of tissue factor/tissue thromboplastin. Tissue factor interacts with factor VII to activate factor X. Here, both pathways share the common pathway and the remaining coagulation cascade continues (Fig. 2*C*).

Question 26. What is the difference between serum and plasma?

ANSWER. Serum is plasma minus its clotting proteins.

Question 27. What are the elementary precautions in the safe handling of blood?

ANSWER. Always wear gloves, safety glasses, and a laboratory coat.

Question 28. Give reasons why typed blood may be still unsafe to transfuse.

ANSWER. Donor blood must be checked for blood-borne diseases (especially HIV and hepatitis B). Blood may only be typed for the more common antigens (A, B, and Rh D). Cross-matching of the donor and recipient blood must be carried out to ensure that the erythrocytes of the donor carry no rarer antigens and therefore cannot be agglutinated. Cross-matching also ensures that the plasma of the recipient carries no antibodies that will agglutinate donor cells.

Question 29. What is the difference between coagulated and agglutinated blood?

ANSWER. Agglutination of blood is an antibody-mediated reaction that occurs in the plasma of the recipient in response to the introduction of foreign antigens on the erythrocytes of the donor. Antibodies have more than one site to bind antigens and can therefore bind many; this leads to agglutination of the erythrocytes, increasing the likelihood of phagocytosis by leukocytes.

Coagulation is a hemostatic mechanism in response to blood loss from a damaged blood vessel. During coagulation, erythrocytes will be trapped together with platelets and plasma in a meshwork of fibrin strands, effectively sealing the leaking vessel and preventing further blood loss.

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DISCLOSURES

AUTHOR CONTRIBUTIONS

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