THE UNIVERSITY OF NEW SOUTH WALES

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BABS3041
(Immunology)

Term 1, 2020

Course Manual

SCHOOL OF BIOTECHNOLOGY AND BIOMOLECULAR SCIENCES

FACULTY OF SCIENCE

COURSE INFORMATION

Units of credit: 6
Pre-requisite(s): (BIOC2101 or BIOC2181 and MICR2011)
or (BIOC2101 or BIOC2181 and BABS2202).

Teaching time: Term 1

COURSE SUMMARY

BABS3041 provides a broad and in-depth coverage of immunology. The course is for students majoring in Medical Microbiology, Immunology, Biotechnology, Biomolecular Science, Medical Science and other areas related to human health who are interested in gaining knowledge in Immunology.

BABS3041 consists of 24 one-hour lectures and 8 three-hour practical classes. The course will first introduce the multiple components of the immune system, their functions, interactions and regulations during immune responses. Then the applied and clinical aspects of immunology will be introduced, including allergy, immunodeficiency, immune system and cancer, vaccination, autoimmunity, engineering antibodies, diagnostic immunology and immunological research strategies. The practical classes introduce students to critical immunological techniques such as immune cell stimulation, immunological assays and flow cytometry. The course also introduces students to critical evaluation of immunological issues of community importance and literature.

COURSE AIMS

This course provides students with a broad and in-depth understanding of the immune system and its functions. It also introduces students to the applications related to the immune system and immunology research.

COURSE LEARNING OUTCOMES (ASSOCIATED ASSESSMENTS)

At the successful completion of this course, the students should be able to:

1. Describe different components of the immune system and their responses to infection and cancer (test 1, test 2 and final exam).
2. Explain how immunological abnormalities may cause human diseases and how immunological interventions may prevent and treat human diseases (test 1, test 2 and final exam).
3. Apply diagnostic laboratory techniques to diagnose immunological disorders (test 1, test 2 and final exam).
4. Plan laboratory experiments and interpret experimental data on research in immunology (the assignment and final exam).
5. Critically evaluate scientific literature in immunology and immunological issues of community importance (the assignment and final exam).

STRATEGIES AND APPROACHES TO LEARNING

BABS3041 uses Blended Learning strategy, combining face to face teaching with online learning materials and activities. The course has been digitally uplifted.

Lectures are delivered face to face and lecture notes are available on the course Moodle website. All lectures are recorded and available via Moodle course site, providing flexibility and further assisting students' learning. Practical classes consist of laboratory-based experiments and computer-based classes. Other on-line learning activities and materials will be provided during the course.

You can access Moodle site for this course via the UNSW website using your UniPass.

TEXTBOOK

The recommended textbook for this course: **Janeway's Immunobiology 9th Edition. Kenneth Murphy. 2017. Garland Science, Taylor & Francis Group, LLC.**

Other recommended book: ***Kuby*** **Immunology 8th Edition. 2019. W.H. Freeman and Company.**

COURSE CONVENER AND LECTURERS

Dr. Li Zhang: Dr Zhang is the course convener and lecturer for BABS3041. Dr Zhang obtained her MB BS degree from Fudan University and PhD degree from the University of Cambridge. She is currently a Senior Lecturer at the University of New South Wales and has been the convener for this immunology course since 2012. Dr Zhang has a long-standing interest in chronic inflammatory diseases. Her previous research was on autoimmune diseases. Since 2008, her group has been research on *Campylobacter* species and their role in inflammatory bowel disease, the genomes and virulence of emerging gut bacterial pathogens, the relationship between gut microbiota and immunotherapy. Dr Zhang can be contacted at: Room 4106, Bioscience South E26. l.zhang@unsw.edu.au

Guest lecturers

Professor Stuart Tangye: Professor Tangye is an immunologist from the Garvan Institute of Medical Research. His research focuses on autoimmune disorders, immunodeficiency, inflammatory diseases and allergic diseases. Professor Tangye delivers the two lectures on B cell and antibody and the two lectures on T cell functions. s.tangye@garvan.org.au

Dr Debbie Burnett: Dr Debbie Burnett is an Immunologist from the Garvan Institute of Medical Research. Her research focus is understanding how the immune system discriminates between self and foreign antigens and how this relates to pathogen defence and autoimmune disease. Dr Burnett delivers one lecture on antigen receptors and signalling. d.burnett@garvan.org.au

Associate Professor Stuart Turville: Associate Professor Turville is from the Kirby Institute, UNSW. His group researches on the mechanisms of HIV spread and using gene therapy to treat HIV infection. Associate Professor Turville delivers the two lectures on immunodeficiency. sturville@kirby.unsw.edu.au

LECTURE OUTLINE

Overview of the Immune System: Different components of the immune system will be introduced.

Innate Immunity (two lectures): The innate immune system will be introduced in these two lectures. Key cells and molecules of the innate immune system will be described.

Adaptive Immunity: This lecture is an introduction to the adaptive immune system. Antigen, B cells, T cells, the clonal selection theory and immunological memory will be introduced.

B cells and Antibodies (two lectures): The structure and function of antibodies will be outlined. The concept of specificity, affinity and affinity maturation will be discussed. The development of B cells and their generation of the diverse antibody repertoire will be introduced.

T cell Function (two lectures): The two lectures introduce various types of T cells, their development and functions.

Cytokines: The general nature of cytokines and the function of selective cytokines will be introduced.

Antigen Receptors and Signaling: This lecture introduces the recognition of antigen by B cell and T cell receptors. Co-receptors and molecules of the downstream signaling pathways will also be introduced.

MHC and Antigen Presentation: The special way in which T cells interact with foreign antigen will be introduced. Students will learn about the Major Histocompatibility Complex (MHC).

Mucosal Immunity: Most infections begin at the mucosal surface and mucosal immune system plays an important role in protecting the mucosal surface. The specific features and function of the mucosal immune system will be introduced.

The Immune Responses to Infection (two lectures): Key defences against different types of infections will be outlined.

Immune Evasion: Strategies that are used by microbes to evade host immune response will be described.

Immunodeficiency (two lectures): Students will be introduced to primary immunodeficiencies that affect different components of the immune system. HIV /AIDS will be the focus of the presentation on acquired immunodeficiency.

Allergic Disease: Allergic disease is a serious problem in the modern world. This lecture will introduce cells and molecules of the Type 1 hypersensitivity reaction.

Vaccination: The history of vaccination will be introduced. Different types of vaccines will be explained, and future challenges for vaccine development will be outlined.

Tolerance and Autoimmunity: The mechanisms by which self-reactivity of B cells and T cells are either eliminated or controlled will be outlined, and autoimmunity will be discussed.

Diagnostic immunology: This lecture will introduce some tests that are used in Diagnostic Immunology.

Immune System and Cancer: In this lecture, you will learn cancers of the immune system and the role of the immune system in controlling cancer.

Engineering Antibodies: The engineering of antibodies is a huge industry. The use of antibodies as biopharmaceuticals and the approaches to antibody engineering will be presented.

Immunological Research Strategies: This lecture will describe several strategies that are used in the studies of immunology.

PRACTICAL CLASSES

ATTENDANCE TO PRACTICAL CLASSES IS COMPULSORY except for Week 4 tutorial

Practical classes will be held on Tuesdays. The contents covered in the morning and afternoon class are same, you need to attend either morning or afternoon class. You will be organized into groups and demonstrators will be assigned.

The specific objectives of the practical classes are:

- To reinforce and better understand information delivered in lectures.
- To provide students with opportunities to explore techniques that are commonly used in immunology research and diagnostic immunology
- Develop experimental and analysis skills
- Apply concepts and skills learned in this course to solve problems

LECTURE TIMETABLE AND LOCATION

Mon 5 -6 pm, Old Main Building 149(K-K15-149)
Wed 1 -2 pm, Mathews Theatre D (K-D23-304)
Thu 1 - 2 pm, Webster Theatre B (K-G15-290)

Lecture	Topic	Date		Week	Lecturer
1	Overview of the Immune System	17/2	Mon	1	LZ
2	Innate Immunity 1	19/2	Wed	1	LZ
3	Innate Immunity 2	20/2	Thu	1	LZ
4	Adaptive Immunity	24/2	Mon	2	LZ
5	B cells and Antibodies 1	26/2	Wed	2	ST1
6	B cells and Antibodies 2	27/2	Thu	2	ST1
7	T cell Function 1	2/3	Mon	3	ST1
8	T cell Function 2	4/3	Wed	3	ST1
9	Cytokines	5/3	Thu	3	LZ
10	Antigen Receptors and Signaling	9/3	Mon	4	DB
11	MHC and Antigen Presentation	11/3	Wed	4	LZ
12	Mucosal Immunity	12/3	Thu	4	LZ
13	Immune Responseto Bacterial Infection	16/3	Mon	5	LZ
14	Immune Response to Viral Infection	18/3	Wed	5	LZ
15	Immune Evasion	19/3	Thu	5	LZ
	Week 6: Flexibility Week				
16	Secondary Immunodeficiency	30/3	Mon	7	ST2
17	Primary Immunodeficiency	1/4	Wed	7	ST2
18	Allergic Disease	2/4	Thu	7	LZ
19	Vaccination	6/4	Mon	8	LZ
20	Tolerance and Autoimmunity	8/4	Wed	8	LZ
21	Diagnostic Immunology	9/4	Thu	8	LZ
	Public Holiday	13/4	Mon	9	
22	Immune System and Cancer	15/4	Wed	9	LZ
23	Engineering Antibodies	16/4	Thu	9	LZ
24	Immunological Research	20/4	Mon	10	LZ

LZ: Dr Li Zhang, ST1: Professor Stuart Tangye, DB: Dr Deborah Burnett,
ST2: Associate Professor Stuart Turville

PRACTICAL CLASS TIMETABLE

Morning groups 10 am - 1 pm. E26 Teaching Lab 12 (K-E26 -1102)
Afternoon groups 2 – 5 pm. E26 Teaching Lab 12 (K-E26 -1102)

Week	**Date**	**Practical class**	
2	25/2 Tue	Cells and tissues of the immune system	
3	3/3 Tue	Tutorial: Tetanus **Test 1**	
4	10/3 Tue	Tutorial: The Immune system in different stages of the human life	
5	17/3 Tue	Cell viability and counting **Test 2**	
		Week 6: Flexibility Week	
7	31/3 Tue	Isolating & activating splenocytes	
8	7/4 Tue	Measurement of IL-2 using ELISA	
9	14/4 Tue	Flow cytometry	
10	21/4 Tue	Tutorial： Clinical cases	

ASSESSMENTS

CONTINUOUS ASSESSMENT (50%)

TEST1 (17.5%) and TEST2 (17.5%)

There will be two tests, which will be held at the start of the practical classes in week 3 and week 5 respectively. Each test is worth 17.5% of your final mark.

Students who miss a test may have the chance to sit a supplementary test, only if they submit a medical certificate or other suitable documents to explain their absence to the course convener by the end of the week of the test. There will only be **ONE** opportunity to sit the supplementary test, which will be held in the week following the test.

ASSIGNMENT (15%)

This year's assignment is related to week 7 and week 8 practical classes. The assignment will be released on Moodle on 30th March. The deadline for submission of this assignment is 3pm, 16th April. There is 10% per day deduction for late submissions. The assignment should be submitted electronically to Moodle site. The hard copy must be submitted to the BABS/BEES/SOMS Student office.

FINAL EXAM (50%)

The final exam is worth 50%, which is scheduled within the exam period at the end of the term. Further information regarding the final exam will be provided in the last lecture.

SPECIAL CONSIDERATIONAND FURTHER ASSESSMENT

Students who believe that their performance, either during the session or in the end of session exams, may have been affected by illness or other circumstances may apply for special consideration. Applications can be made for compulsory class absences such as (laboratories and tutorials), in-session assessments tasks, and final examinations. **Students must make a formal application for Special Consideration** for the course/s affected as soon as practicable after the problem occurs and **within three working days of the assessment to which it refers**.

Students should consult the "Special Consideration" section of Moodle for specific instructions related to each BABS course they are studying. Further general information on special consideration can also be found at https://student.unsw.edu.au/special-consideration.

HOW TO APPLY FOR SPECIAL CONSIDERATION

Applications must be made via Online Services in myUNSW. **You must obtain and attach Third Party documentation before submitting the application. Failure to do so will result in the application being rejected.** Log into **myUNSW** and go to **My Student Profile tab > My Student Services channel > Online Services > Special Consideration.** After applying online, students must also verify supporting their documentation by submitting to UNSW Student Central:

- Originals or certified copies of your supporting documentation (Student Central can certify your original documents), and
- A completed Professional Authority form (pdf - download here).

The supporting documentation must be submitted to Student Central for verification **within three working days** of the assessment or the period covered by the supporting documentation. Applications which are not verified will be rejected.

Students will be contacted via the online special consideration system as to the outcome of their application. Students will be notified via their official university email once an outcome has been recorded.

SUPPLEMENTARY EXAMINATIONS

SUPPLEMENTARY EXAMINATIONS:

The University does not give deferred examinations. However, supplementary exams may be given to those students who were absent from the final exams through illness or misadventure. Special Consideration applications for final exams and in-session tests will only be considered after the final exam period when lists of students sitting supplementary exams/tests for each course are determined at School Assessment Review Group Meetings. Students will be notified via the online special consideration system as to the outcome of their application. **It is the responsibility of all students to regularly consult their official student email accounts and myUNSW in order to ascertain whether or not they have been granted further assessment.**

The supplementary exam period for T1 2020:

25 May –29 May

Supplementary exams will NOT be offered on any alternative dates.

PLAGIARISM

Plagiarism is using the words or ideas of others and presenting them as your own. It can take many forms, from deliberately cheating to accidentally copying from a source without proper acknowledgement. If any student is caught plagiarizing in this course. they will receive no marks for that course task. For further information on plagiarism, the university Learning Centre has substantial information on the subject including material on how to avoid plagiarism. Information can be found at: www.lc.unsw.edu.au/plagiarism.

LAB RULES & SAFETY

Biological laboratories are inherently dangerous places and it is the responsibility of everyone who works in the laboratory to ensure that the risks are minimized. It is your responsibility to ensure that you come to labs prepared and work in a manner that will not put yourself or others in danger. Risks related to each practical class are outlined individually. Some general laboratory rules are outlined below.

- Wear a fastened lab coat.
- Wear disposable gloves when handling samples and other experimental materials and dispose the gloves if they are contaminated.
- Dispose all materials in the appropriate bins. All contaminated waste must go in the biohazard bags provided. Broken glass must go in the broken glass bin and anything sharp in the sharps bin.
- Wear fully enclosed shoes, preferably made of a sturdy material.
- Keep all hair tied back.
- Move calmly and slowly around the lab.
- Keep only what is necessary on your desk. All other things should be kept in the cabinets provided.
- Wipe down benches, before and after the practical class.
- Act maturely and responsibly.
- Do not eat, drink, apply makeup or chew gum (or pens). Any food or drink you have must be stored inside your bag at all times during the lab.
- Do not invite any one into the lab. Only the people who are meant to be in the lab should be there.
- Keep all experimental reagents and equipment covered. Never leave things such as pipette tips etc lying around uncovered unnecessarily.
- Never attempt to recap a needle.
- Label all samples with your name, date, demonstrator and what kind of sample it is.
- Never handle ‘clean items’ such as your bag whilst wearing gloves. Even if you think your gloves are clean, they may not be.
- Wear safety goggles when handling things that may splash into your eyes.

BIOLOGICAL HAZARDS

- All biological samples are potentially harmful if ingested or exposed to body surfaces. Ensure that you wash your hands thoroughly with an anti-microbial detergent and dry them thoroughly before leaving the lab.
- All specimens of cells, tissues or body fluids from humans or animals are potential sources of infection. Every effort is made to ensure that any specimens you receive are not infectious. However, the use of universal precautions when handling specimens is essential. The main principle of universal precautions is to assume everything is a source of infection.
- Always wear gloves, a lab coat, closed toe shoes and when there is a chance of splashing, safety goggles. keep your lab coat in a plastic bag after classes to avoid spreading any potential contamination.

CHEMICAL HAZARDS

Most buffers and media used in the course are not or low hazards at the concentrations used, however all chemicals should be considered potentially hazardous.

PHYSICAL HAZARDS

Common sense precautions must be observed when dealing with heat sources such as Bunsen burners water baths as well as sharp objects.

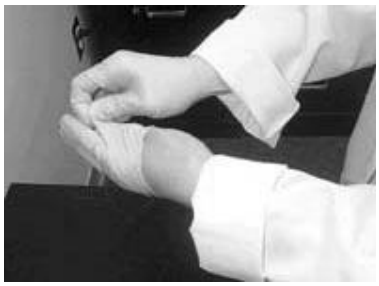
IN CASE OF EMERGENCY

- If there is an accident with a biological sample, try to mop up what you can and call a fellow student to get a demonstrator to help. Do not move yourself in case you spread the contamination further.
- If there is a fire, remove yourself from immediate danger and call someone in authority to help.
- Call a demonstrator immediately if anything happens. Do not try and deal with the situation yourself.
- If you splash something in your eye, flush it out at an eye station immediately and ask someone to call a demonstrator for help.
- If there is a medical emergency call a demonstrator for help, and then try and ascertain what caused the emergency (e.g. leaking gas tap, exposed power cable) and rectify the situation if you think you can do so safely. If not, wait for your demonstrator.

SAFELY REMOVING GLOVES

In the immunology lab, we often wear gloves to protect ourselves from the substances that we are working with. However, this only works if we can safely remove those gloves without contaminating our hands (photos used with

permission from the University of Maryland, Department of Environmental Safety).

	Pinch one glove in the palm and pull towards the ends of the fingers until the gloves folds over.
	Keep pulling so that the fingers are inverted.
	Scrunch the removed glove in the palm of the other gloved hand.
	Now, slide your index finger underneath the remaining glove.
	Pull the glove down over the fist.

	Touch only the inside of the gloves and drop into the appropriate container. Wash hands thoroughly.

HEALTH AND SAFETY PRECAUTIONS FOR ELECTRONIC DEVICES INCLUDING LAPTOP COMPUTERS AND MOBILE PHONES

Mobile phones: For your own safety when using your mobile phone in class please ensure it is placed in a plastic zip lock bag. Every student will be provided with a zip lock bag in their first practical class which they should continue to use during the session (keep it with your lab book). If you misplace or loose this bag you will be expected to provide your own zip lock bag.

Computers and tablets: this applies to either your own device or those supplied by the school. Please cut a section of benchcoat (this will be provided in the lab class) and place your device on this on the lab bench to separate it from your other laboratory work. DO NOT wear gloves when using these devices. If the lab is being used as a dedicated DRY LAB (please check signage on the door) you will not be required to do this as the benches will be cleaned and decontaminated before your class.

RISK ASSESSMENTS

You will be required to complete Risk Assessment forms before wet laboratory classes.

WEEK 2 CELLS AND ORGANS OF THE IMMUNE SYSTEM

AIMS

- To familiarize students with the preparation of a stained blood smear
- To develop your ability to identify different types of leucocytes in the blood
- To better understand the anatomy of the immune system

INTRODUCTION

In this practical class, you will prepare a blood smear and use it to learn how to identify the major cell types of the blood. You will also perform a differential cell counting to quantify the relative proportions of different immune cell types in the blood. Differential cell counting is a standard clinical test, in many clinical conditions the relative proportions of different cell types change.

You then examine the structure of some lymphoid organs, which will help you to better understand the anatomy of the immune system.

At the end of this practical class, you should have a good understanding of the common immune cells and the anatomy of lymph nodes, the spleen and the thymus.

RISK ASSESSMENT

This practical class involves the use of horse blood, which is commercially purchased. However, you should treat all blood samples as potentially infectious. You must also remember that there may be unidentifiable pathogens in the blood. These issues must guide your risk assessment and the procedures you follow in the practical class to minimize these risks.

ACTIVITIES

- Preparation of a blood smear
- Staining the blood smear
- Differential blood leukocyte count
- Examination of the structure of lymphoid organs

PREPARATION AND STAINING A BLOOD SMEAR

REAGENTS & EQUIPMENT

- Horse blood
- Glass microscope slides
- Benchkote paper
- Biohazard waste disposal bag
- Biohazard sharps disposal bin

- Disposable gloves
- Paper towel
- Coplin jars with all solutions for the Wright's stain
- Pipettes and pipette tips

PREPARATION OF A BLOOD SMEAR

All students should make a blood smear with the horse blood provided.
You should prepare your slides as follows:

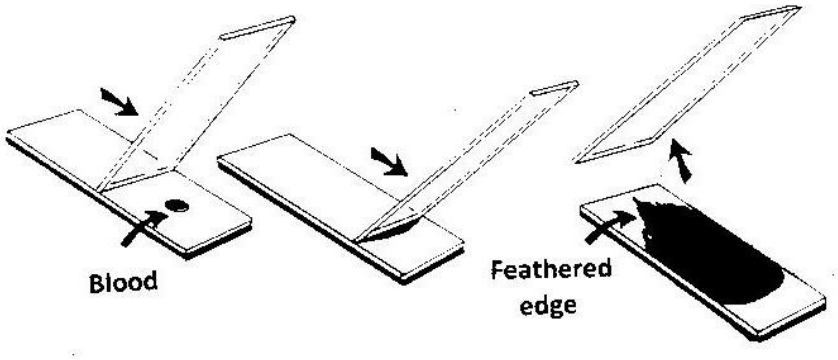
1. Using a pipette, place a 5-10µl drop of blood on one slide, towards one end**.**

2. Draw a second 'spreader' slide back until it touches the drop. The slide should be held at approximately a 45º angle (see figure below).

3. Allow the blood to capillarise between the slides. That is, the blood should spread out across the slide at the interface of the two pieces of glass

4. Smoothly and quickly push the 'spreader' slide across the base slide to form a smear of blood.

5. Air dry



Method of preparing a blood smear

STAINING A BLOOD SMEAR

1. Fix the slide by submerging in 100% methanol for 1 min. Drain well by touching the end of the slide on a paper towel and then let it dry.

2. Place the slide into Wright's stain diluted in methanol for 1 minute. Drain well by touching the end of the slide on a paper towel.

3. Place the slide into Wright's stain diluted in phosphate buffer for 8 min. Drain well by touching the end of the slide on a paper towel.

4. Gently wash by dipping the slide in water repeatedly for 30 sec. Drain well by touching the end of the slide on a paper towel.

5. Differentiate by placing the slide for 30 sec in phosphate buffer. Drain well by touching the end of the slide on a paper towel.

6. Blot the bottom of the slide (the unsmeared side) and air dry for 5-10 min.

DIFFERENTIAL BLOOD LEUKOCYTE COUNTS

1. Place the blood smear on the microscope stage and examine the smear under low power (10x then 40x) to identify the 'feathered region' (see diagram above) where cells should be neither too few nor too many. The area immediately beneath the feathered edge will give you the most accurate count.

2. Using the oil immersion lens (100x), examine a number of white cells. Make a drawing of each different morphological type of white cell that you see and identify them, by reference to the images above.

3. When you feel confident that you can identify the different types of cells, examine exactly 100 white cells, by working systematically across the slide. Allocate each cell to one or other type and estimate the percentage of each cell type in the smear. This is known as a 'Differential White Cell Count'.

4. Record your results in the table below

RESULTS & WORKING

FILL OUT THE TABLE BELOW:

	Monocytes	Lymphocytes	Eosinophils	Basophils	Neutrophils
Your count					
Partner's count					
Total					
Percentage of cells counted					

IN THE SPACE BELOW, DRAW EACH CELL TYPE AND LABEL THEIR SPECIAL FEATURES:

LYMPHOID ORGANS

Each group will be provided with diagrams of structure organization of lymph nodes and spleen.

1. What are the functions of these lymphoid organs?

2. Write down the key structure features of each lymphoid organ

3. Compare the structure organization of lymph nodes and spleen, can you identify their common and distinct features? Can you relate these features to the functions of these lymphoid organs?

Each group will also be provided with stained sections of rat or mouse lymph nodes and spleen.

1. Compare the structure diagrams of lymph nodes and spleen with their stained sections. How many of the key features shown on the diagrams can you identify in the stained sections?

WEEK 3 TETANUS TUTORIAL

This tutorial was developed from materials originally written by Parker A. Small Jr., Edwin Blalock and Susan M. Johnson of the University of Florida.

ACTIVITIES

1. Read the basic information of Tetanus provided in this tutorial.
2. Form groups of four, answer the questions in part 1 and part 2 (group discussion is strongly encouraged).
3. Your tutor will ask each group to present their answers.

The following concepts should be clarified through this tutorial.

a. Antibody vs. antigen

b. Innate (non-specific) vs. acquired (specific) immunity

c. Cell mediated vs. antibody-mediated immunity

d. Active vs. passive immunity

e. Primary vs. secondary immune response

f. Toxoid vs. toxin

TETANUS - THE BASIC INFORMATION

Tetanus is caused by the bacterium *Clostridium tetani* which is found widely distributed in the environment, e.g. dust, soil. It is also found in the mouths and intestinal tracts of many animals, so tetanus may result from animal bites or from extensive exposure to manure. The bacterium is very resistant and can survive for years in the environment. The disease is characterised by generalised muscular spasms and seizures. These develop 3 to 21 days after exposure, depending upon a range of factors including the size of the inoculum (the number of organisms that enter the body). Tetanus has a very high mortality. Immunisation results in almost 100% protection and so there is no excuse for any cases in Australia. Are you immunised? When were you immunised?

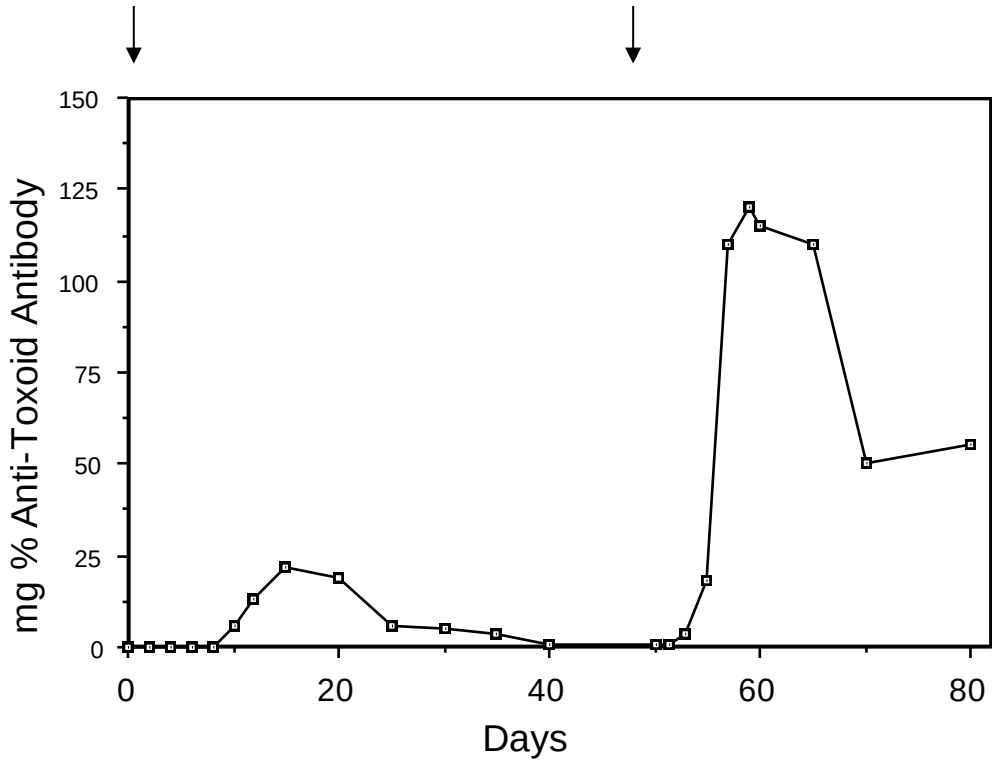
The organism usually enters the body via trauma, e.g. standing on a rusty nail, motor mower or car accidents, etc. Soil and manure can be heavily contaminated with the organism. *Clostridium tetani* does not like oxygen and so grows in the body when the blood flow is damaged and/or reducing agents are present. If these conditions are met, e.g. at the tip of a rusty nail, the organism multiplies and produces a protein toxin, **Tetanus toxin** which interferes with neuromuscular function. This toxin causes all the significant disease symptoms. If you can neutralise the action of the toxin then tetanus will not result - even if the organism is still present in the tissues. To teach the body how to quickly neutralise the toxin, tetanus vaccination was developed. Obviously, we cannot use toxin as the vaccine, hence the use of toxoid. Toxoid is the purified protein toxin of *Clostridium tetani* that has been treated with formaldehyde. This destroys the toxic activity, but the ability of the protein to generate an effective anti-toxin response is retained.

If someone is judged to be in danger of tetanus, they may be injected with tetanus immunoglobulin that has been purified from the serum of individuals with high concentrations of immunoglobulin to tetanus toxin. This is called passive immunisation. In the past, human antibodies were unavailable, and horse "antiserum" - that is, serum that contains appropriate antibodies - was used. **Note that horse antiserum is still used to treat snakebite.** Why do you think this might be so?

ANSWER THE FOLLOWING QUESTIONS

PART 1: CHOOSING THE **ONE** CORRECT OR MOST APPROPRIATE ANSWER.

1. Judging from the following graph, how many days does it take to detect specific antibody in the serum after a primary and a secondary immunization respectively? The arrows indicate the times when antigen injections were given to the patient. "mg% Anti-Toxoid Antibody" refers to the number of mg of antibody detected in 100 ml of the patient's serum.



(a) 15 and 10
(b) 10 and 15
(c) 10 and 4
(d) 4 and 10
(e) 3 and 3

2. Bacteria on your skin will not usually produce disease. The immunity to bacteria and other micro-organisms provided by intact skin is:

(a) specific and innate.
(b) acquired and antibody-mediated.

(c) innate and non-specific.
(d) acquired and cell-mediated.
(e) none of the above.

3. A twelve-year old car accident victim with severe, dirty wounds received when she was thrown clear of the vehicle, was just brought in. Her parents state she has not had previous immunization against tetanus. As protection against the possibility of tetanus now and in the future, the preferred method of treatment would be:

(a) a mixture of toxoid and antitoxin as one injection
(b) an antitoxin injection this visit.
(c) a toxoid injection this visit, with an injection of antitoxin approximately three weeks later.
(d) separate injections of toxoid and antitoxin in different sites on this one visit, thereby insuring active and passive immunization.
(e) none of the above.

4. Tetanus toxoid:

(a) is an active toxin.
(b) cannot react with antibody to tetanus toxin.
(c) retains antigenicity for immunization purposes.
(d) is chemically identical to tetanus toxin.
(e) stimulates antibody formation to the organism, *Clostridium tetani.*

5. Specific antibody concentrations in a typical memory response differ from those seen in a primary response, being:

(a) more prolonged, but being neither higher nor occurring faster.
(b) higher but occurring no sooner.
(c) no high but occurring sooner.
(d) higher and more prolonged, and occurring sooner.
(e) higher but less prolonged, and occurring sooner.

6. A 25-year-old patient has a clean wound from broken glass, but she received a full course of the tetanus vaccine as a child, and a booster vaccination seven years earlier. Which of the following statements is correct and provides the best treatment strategy?

(a) Exposure to bacterial toxin will activate memory cells, leading to the development of protective antibodies.
(b) As the wound is relatively minor and clean, and the patient is an adult with a complete vaccination history, and a booster shot seven years ago, there is no need for treatment
(c) A booster shot of toxoid will fail to provide protective immunity because of the loss of memory cells after the passage of seven years. Passive immunization is therefore necessary
(d) A booster of shot of toxoid will stimulate long-lived memory cells, which will generate a protective antibody response.
(e) Passive immunization with tetanus immunoglobulin will provide short term protection, and immunization with toxoid will activate long term protection by the stimulation of memory B cells.

7. Serum sickness is a condition when massive amounts of antigen combining with equally massive amounts of specific antibody. This results in the formation of more immune complexes than cannot be handled by the body's clearance mechanisms. Serum sickness can be a major problem with which of the following procedures?

(a) active immunization against tetanus.
(b) passive immunization against tetanus using heterologous immunoglobulin.
(c) passive immunization against tetanus using homologous immunoglobulin.
(d) none of the above.
(Homologous means made in the same species, in this case - human, while heterologous means made in a different species, eg. horse)

8. A patient who needs passive immunization for snakebite but the only available antibody comes from horses. The following important aspect must be taken into account before administering the horse antibody:

(a) the patient's blood type.
(b) history of previous passive immunizations.
(c) history of active immunizations.
(d) the snake's blood type.
(e) the antibody half-life, passive immunization should be repeated every six to eight months if protection is to be maintained.

PART 2: ANSWER THE QUESTIONS RELATED TO FOUR CLINICAL CASES. TICK ONE OR MORE ANSWERS AND BRIEFLY WRITE THE REASONS FOR YOUR CHOICE IN THE SPACE PROVIDED.

Case 1: Dermontt Tan, a 31-year-old man, who as a soldier four years before, had received all common vaccinations and a complete course of tetanus immunisation, presents himself at your office with a one-inch laceration of his right forearm caused by a dog bite right through his wool shirt. Fortunately, in Australia at present, thanks to the success of quarantine, we do not have rabies (a viral infection). But your real concern is the possibility of other infections, and especially tetanus, since that is the primary life-threatening consequence of the event. What are you going to do about it?

(1). Give patient tetanus toxin.

(2). Give patient tetanus toxoid.

(3). Give patient human anti-tetanus immunoglobulin.

(4). Do nothing and let the patient's immune system deal with the problem.

Case 2: Crystal Sharp, a 5-day-old baby has been brought to your office by his mother, a nurse who has just returned from overseas. Mother and baby have flown in from Africa where the baby was born. The mother is concerned because the baby was born in a village where an African friend, following local custom, coated his umbilicus with cow manure. She is concerned about neonatal tetanus. What are you going to do?

(1). Give patient tetanus toxin.

(2). Give patient tetanus toxoid.

(3). Give patient human tetanus immunoglobulin.

Apart from administration of tetanus immunoglobulin, how else might Crystal receive passive immunization?

Case 3: Rosario de la Cruz, a 35-year-old woman, who is a recent migrant to this country, presents herself at your office with a three-inch jagged wound to her head after a nasty fall in the bush. A year ago, she fell off a tractor while tilling a field and received a six-inch laceration of her left thigh. At that time, she was given horse anti-tetanus serum because she had never been immunized against tetanus, and because in the remote area of the country where she lived, human antiserum was unavailable. She left town before her local physician could immunize her with tetanus toxoid. What do you do to prevent tetanus in this patient?

(1). Give the patient tetanus toxin.

(2). Give the patient tetanus toxoid.

(3). Give the patient horse tetanus immunoglobulin.

(4). Give the patient human tetanus immunoglobulin.

(5). Do nothing, because she will still be protected by the previous injection of anti-tetanus serum.

Note: Horse antiserum is no longer used in Australia to prevent the development of tetanus. Before the development of economical serum separation techniques, and the setting up of large blood bank facilities, companies immunized horses because they were easy to bleed and gave a very high yield of serum antibody. However, for the purpose of this teaching exercise, it is useful to consider that this product is still available.

Case 4: Brian Bingham, a 21-year-old man who works in a reptile park presents himself at your office with a double puncture wound in his foot. He reports that he has just been bitten by a snake, which he then produces from his bag (Fortunately it is already dead!). It is a brown snake. Your patient is in luck, because in your fridge, you just happen to have the crucial antivenom. The antivenom - serum containing anti-venom antibodies - was raised in a horse. Brian studied Immunology last year at UNSW, and he is worried that he will mount an immune response to the horse proteins. He refuses treatment unless you provide him with human antibodies. You point out that none are available, but he refuses to believe you, insisting that he is being discriminated against as a public patient. Brian dies.

1. Why does Brian die? (The answer is not immunological.)

2. Why is human snake antivenom unavailable?

3. How would you prepare anti-venom antibodies in a horse?

4. If Brian received the antivenom and survived, how would you treat him if he was bitten by a brown snake again?

WEEK 4 TUTORIAL: THE IMMUNE SYSTEM IN DIFFERENT STAGES OF THE HUMAN LIFE

INTRODUCTION

The ability of the immune system in performing its function varies in different stages of human life. A fetus is in a relatively sterile environment *in utero.* After birth, the newborn is rapidly colonized by many different commensal microbes and may also be exposed to various pathogens. The first major exposure to microbes is during passage through the birth canal and then by oral, skin and respiratory contact with the exterior. These microbes and other environmental factors encountered in human life continuously shape the immune system. For this tutorial, you should aim to understand the changes of the immune system in different stages of the human life and appreciate that many environmental factors may affect the development and functions of an individual's immune system.

ACTIVITIES

Students form groups of four to work through different scenarios. Try to answer all questions and group discussion is greatly encouraged. After completion of questions in each scenario, tutor-group discussion will be organized by your tutor.

Scenario 1: Influenza is an infectious disease caused by an influenza virus. There are several different types of influenza viruses that can cause human infection and the most common infection in human is caused by influenza A. Upon detection of influenza viruses, the immune system defends the host using various mechanisms such as producing cytokines, producing specific antibodies and T cells. In most of the cases, the immune system successfully clears out the viruses and patients recover. However, the infection in some individuals may be more severe and sometimes may cause death.

Figure 1 (data adapted from reference 1) shows the death rate caused by influenza in different age groups and the changes of immune response to influenza over the lifetime of an individual. Observe Figure 1 and answer the following questions.

Reference:

1. Simon, A.K., G.A. Hollander, and A. McMichael, *Evolution of the immune system in humans from infancy to old age.* Proceedings of the Royal Society B: Biological Sciences, 2015. **282**(1821): p. 20143085.

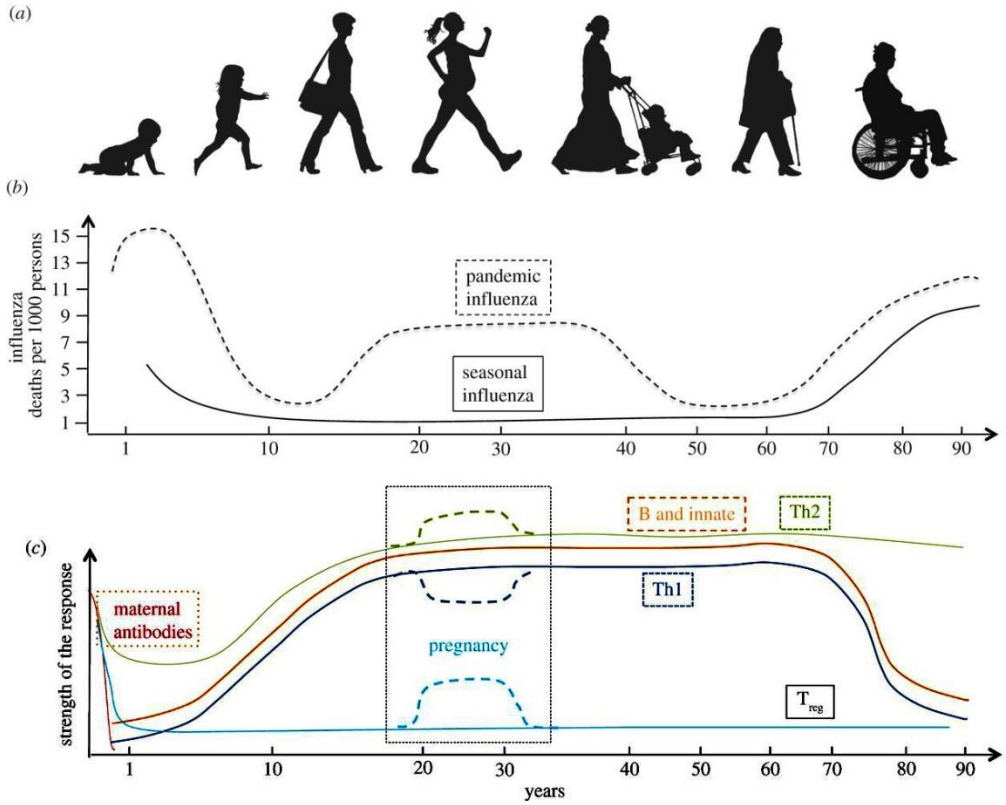


Figure 1 Death rate caused by influenza in different age groups and the changes of immune response to influenza over the lifetime of an individual (1)

1. Which age groups have higher death rates caused by influenza?

2. Summarize the immunity during the first year of human life (from newborn to one year old)

3. Compare the immune response of an individual in childhood, adult period and old age.

4. Which age groups tend to have Th2 immune response?

5. Compare the immune response to influenza virus in pregnant and non-pregnant women, identify the differences and suggest reasons for these differences.

6. Can you explain the immunological reasons why the death rates caused by influenza are higher in some age groups?

Scenario 2:

Peter and John are identical twin. After they were born, their mother found that her milk was not enough for both babies, so she breast-fed Peter but gave John formula-feeding. Both Peter and John grew well, however the mother noted that John got sick more often with gastrointestinal and respiratory infections than Peter.

1. Can you suggest some reasons why Peter had better resistance to infections than John?

2. Using the laboratory computers to perform an internet search to find out what immune components are present in human breast milk. List the information you have found in the space below.

Scenario 3: Two groups of genetically identical mice were raised under different conditions: Group 1 mice were raised under sterile conditions (germ-free) and group 2 mice were raised under conventional conditions. When challenged with enteric pathogens such as *Shigella flexneri* and *Salmonella typhimurium*, germ-free mice showed decreased resistance to infection and increased mortality as compared to mice raised under conventional conditions.

1. Using the laboratory computers to perform Google search to find out evidence that gut microorganisms affect the development and functions of the immune system. List the research articles and their main findings in the space below.

2. Discuss the reason why germ-free mice had decreased resistance to infection.

WEEK 5 CELL VIABILITY AND COUNTING

AIMS

- To accurately determine the concentration of cells in a given suspension
- To accurately determine the viability of a given suspension of cells.
- To be able to readily perform calculations involving cell counts, viability and concentrations.

INTRODUCTION

Counting cells to determine viability and concentration is one of the most frequently performed tasks in immunology. It may seem laborious to you now, but with practice you will find that it becomes a task you can complete in a few minutes. In this exercise you will be working individually. Due to lack of resources however, you will be taking it in turns to count cells and to answer the questions provided.

RISK ASSESSMENTS

In preparing your risk assessment, you should note that you will be unsure of the origin of the cell samples you will use, and you should therefore treat them as though they are potentially infectious. That means that universal precautions must be observed, including the wearing of latex gloves.

REAGENTS & EQUIPMENT

Each demonstrator group will be provided with the following:

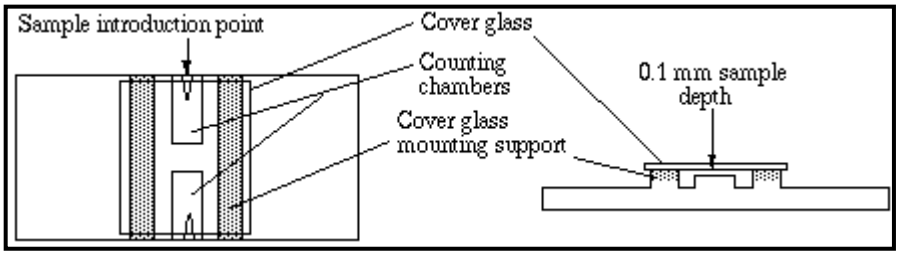
- Haemocytometers and coverslips
- Trypan blue solution
- PBS
- Pipettes and pipette tips.
- 1.5ml tubes
- Microscopes
- Tally counter
- Samples 1 & 2
- Ice

PROCEDURE

1. You are provided with two samples. You will work with them in turn, and perform a cell count as outlined below.
2. Pipette 20µl of sample into a 1.5ml tube.
3. Add 20µl of Trypan Blue. Close the tube and flick it gently to mix. Only add the trypan blue if you are ready to count the cells. Trypan Blue can be

quite toxic to cells and cause cell death if the dye and sample are kept in contact for too long.

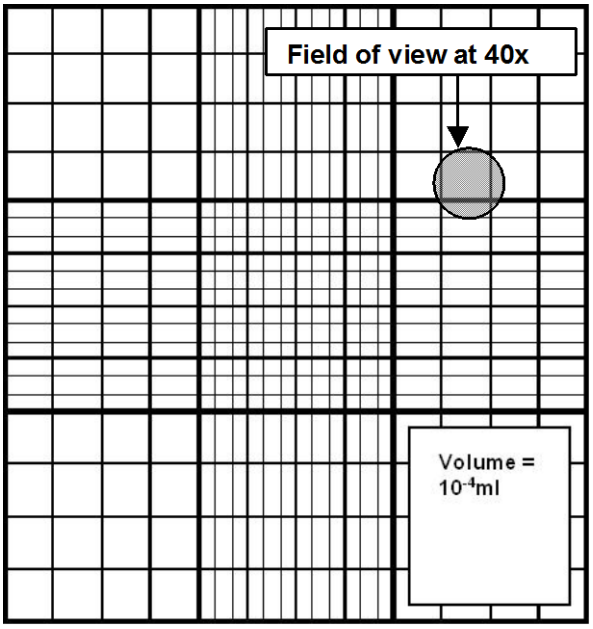
4. Put the coverslip on the haemocytometer and add 10μl of sample to the well by pipetting a drop in the V shaped groove at the edge of the coverslip and allowing capillary action to draw the sample into the well. Seek help from your demonstrator if you have problems. Be sure to use the coverslip that came with your haemocytometer, as ordinary coverslips are smaller and thinner and do not fit correctly.



Schematic diagram of a haemocytometer with coverslide. Image reproduced with permission from D. Caprette. Rice University.

5. Place on the microscope stage and using the 4x objective lens, focus on the large square. Swing in the 10x objective and then the 40x objective to reach suitable magnification for the count. This magnification is needed to allow you to distinguish cells from non-cellular debris.

The haemocytometer has a grid that is illustrated below and described on the next page.



Each large square has an area of 1mm x 1mm, and when the coverslip is applied to the hemocytometer, a space is created with a depth of 0.1mm. As the volume of $1mm^3$ is 1 microlitre, the volume that sits above the square therefore has a volume of 0.1 □l or 10^{-4}ml.

6. As you know the volume that sits above each square, you can count cells and then calculate the concentration of the cells in the added solution. How many squares you count depends on how many cells are present. Accurate counts generally require about 100 cells to be counted. Generally you will count about 100 cells if you count the cells in the four large corner squares. If there are not enough cells, you could count all nine squares, or prepare a new cell preparation with trypan blue that is not diluted with PBS. If there are too many cells, you could count four of the sixteen smaller squares in each of four large squares, or increase the dilution when preparing another cell suspension.

   Accurate counting requires you to deal with cell clumps. If you see lots of clumps, try vigorously pipetting the sample up and down before loading, to try and shear these clumps apart.

7. Once all squares are tallied the following calculation is performed:

   z / (No. large squares counted) x (dilution factor) x 10^4 cells/ml

In this case z = count of cells, 4 large squares were counted and the dilution factor = 2

Example: If z = 144

$$(144/4) \text{ cells } x2\ x10^4 = 7.2 \times 10^5 \text{cells/ml}$$

If you then need to make up your cells to a particular concentration, standard volume and concentration formulae apply.

$C_1 V_1 = C_2 V_2$ where C= Concentration (cells/ml) and V= volume (ml).

The viability of the cells can be worked out as well, as dead cells are stained blue by the Trypan Blue, whereas live cells do not take up the dye. To work out the viability of the cells, another simple calculation is performed:

Viability % = [Live cells/Total number of cells (live + dead)] x 100

Example: If we counted 126 live (clear) cells and 18 dead (blue) cells

Viability = [126/144] x 100 = 87.5%

Rinse the haemocytometer and coverslip with running water and dry with Kimwipes™. Do not use any other tissue to clean the slide, as the small amounts of lint will block the channels on the haemocytomer.

RESULTS & WORKING

Sample No 1.

No. Cells Alive: ___

No. Dead Cells: ___

Dilution Factor: ____

Cell concentration:

What is the cell viability?

If the sample was taken from an original sample of 10mls, how many cells were in the original sample?

Sample No 2.

No. Cells Alive: ___

No. Dead Cells: ___

Dilution Factor: ____

Total cell concentration:

What is the cell viability?

If the sample was taken from an original sample of 10mls, how many cells were in the original sample?

QUESTIONS

From an initial cell culture volume of 15mls, 50µl were taken and 171 live cells and 28 dead cells were counted in 2 squares of a haemocytometer with a dilution factor of 2. You want to set these cells up in new culture media at a live cell concentration of 5 x 10^5 cells/ml and continue to grow them. Calculate:

The concentration of all cells

The viability of the cells

The number of cells in the initial culture

The volume of culture media you will need to make up a new 15ml culture.

From an initial culture of 20mls, 241 alive and 44 dead cells were counted from 2 squares with a dilution factor of 10. You need to make 15mls of cell suspension with a total cell concentration of 2.5 x 10^7cells/ml cells for your experiment.

Write down the steps that are needed to prepare such a cell suspension.

You have 2mls of a cell suspension that is at a concentration of 3 x 10^6cells/ml. What volume of the original suspension would you add to what volume of buffer to give you 10mls of suspension at a concentration of 0.25 x 10^6cells/ml?

You have 5mls of a cell suspension at a concentration of 1 x 10^7cells/ml. You are performing an experiment where you will need a range of concentrations between 5 x 10^6cells/ml and 5 x 10^4cells/ml. You decide the best way to do this is through doubling dilutions of your cell suspension. i.e. each step in your range will be half the concentration of the previous step. Work out a simple way to make a series of doubling dilutions of your original cell suspension, in Eppendorf tubes, giving 0.5ml of each concentration.

WEEK 7 ISOLATING & ACTIVATING SPLENOCYTES

AIMS

- To dissect a mouse, identify tissues of the immune system and remove the spleen.
- To prepare a sterile single cell suspension from the spleen.
- To compare the results of activating spleen cells with an antigen and with a mitogen, and to compare the response of normal and transgenic animals.

INTRODUCTION

Being able to perform surgical procedures and create sterile cell suspensions is important in immunological research. Normally, when trying to obtain sterile cell suspensions, we work within a laminar flow cabinet (see diagram below). Such cabinets protect workers from potentially hazardous cells and organisms, but they also separate cells from the laboratory environment, with all its contaminating microorganisms. We do not have the facilities, however, for every group of students to work within a laminar flow cabinet. Today's experiment will therefore be performed on the laboratory bench. Liberally use 70% ethanol to thoroughly cleanse the equipment, bench and your gloves when performing this procedure. This treatment should hopefully reduce the load of bacteria and fungi.

The cell culture media you will use today is made from:

Dulbecco's Modified Eagle Media (DMEM): an isotonic media that has been for cell culture. It is light red in colour due to the presence of a pH indicator that goes yellow when acidic and red when basic.

10% heat inactivated fetal calf serum: provides essential amino acids and other factors necessary for cell growth. It has been heat inactivated at 56^{o}C for 30 minutes. This inactivates complement proteins that could otherwise 'attack' the cells in culture.

2mM L-glutamine: this is an essential amino acid that is very unstable and needs to be replenished in the media.

100U/ml Penicillin and Streptomycin: these are included to help inhibit the growth of bacteria that may contaminate the media.

2.5µg/ml Amphotericin B: this is an antimycotic agent to reduce the risk of fungal contamination.

Two kinds of mice will be used in this experiment. T Cell Receptor (TCR) transgenic mice will be used on Tuesday, and normal BALB/c mice will be used on Wednesday. The transgenic mice have had their TCR specificity altered so that 50-80% of the TCRs in the mice are specific for a single protein: moth cytochrome C.

RISK ASSESSMENT

This laboratory class involves the dissection of animals and the preparation of a suspension of mouse leucocytes. While every effort is made to ensure that the animals supplied do not pose a risk of human infection, this cannot be guaranteed. Thus, all procedures involving the handling of animals and cells MUST be carried out using universal precautions.

Study the diagram below, and answer the following questions. Note that the lymph nodes are shown much larger than they normally appear in a mouse -even an infected mouse. It may help to also consult the photograph that will be available in the class.

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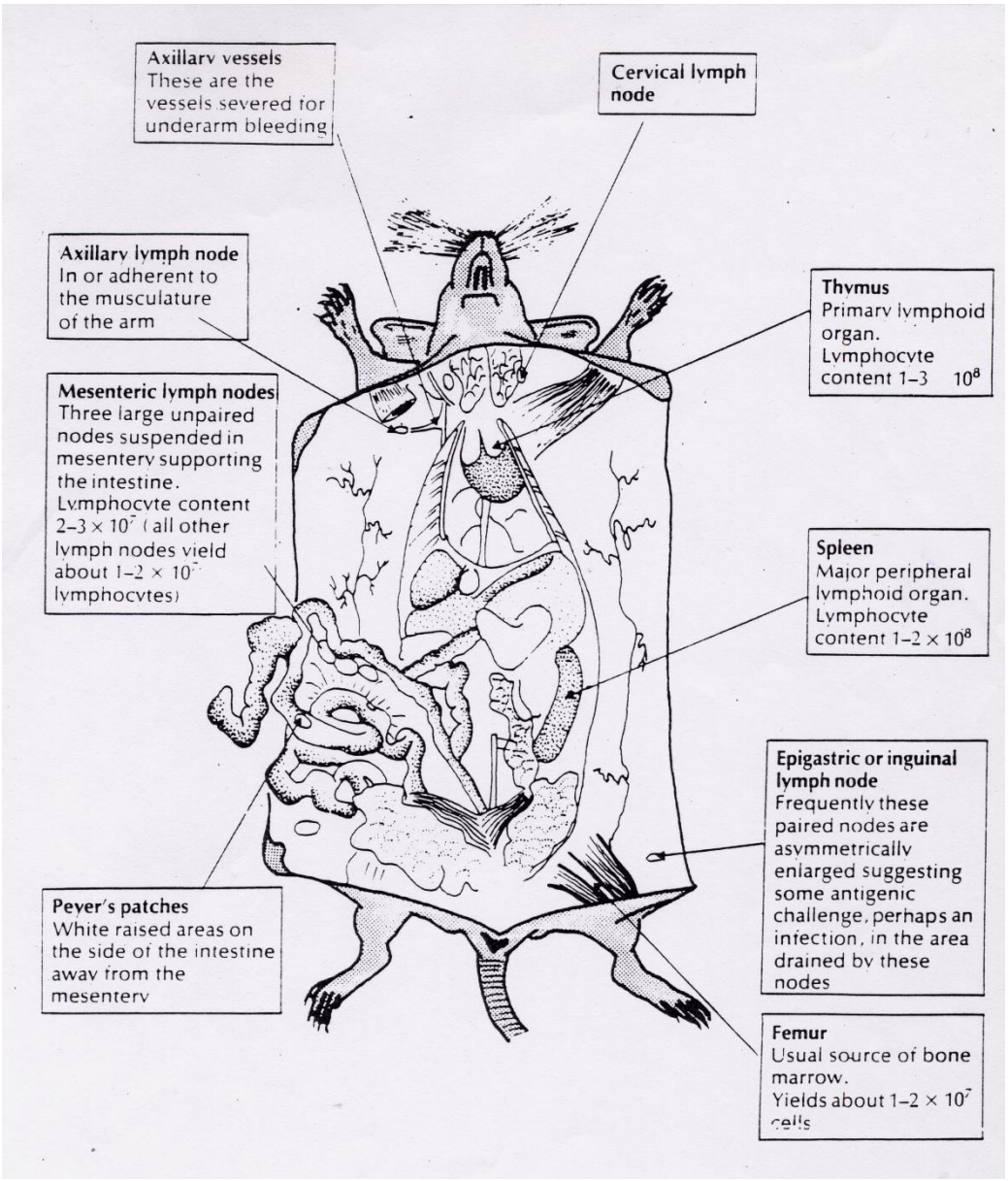


At the end of this practical, you must be able to identify the following:

The spleen

The mesenteric lymph nodes

The thymus

The Peyer's patches

Reagents & Equipment

- Sterile PBS
- 4mls of filter sterilized Red Blood Cell Lysis Solution
- 70% Ethanol (squeeze bottle)
- DMEM based Cell Culture Media (CCM)
- Concanavalin A (ConA) – a T cell mitogen
- LPS – a mouse B cell mitogen
- MCC antigen
- Ovalbumin (OVA) antigen
- Sterile Petri dishes
- 5ml syringes
- 50ml tubes
- Sterile scissors and forceps
- 200ml beaker filled with 70% ethanol

PROCEDURES

For this practical you will have one mouse per group of 4 students. You will harvest splenocytes as a group, before finally setting up cultures of cells on a 96-well plate. You will share this plate with other students.

Your demonstrator will guide you through the following procedures.

1. Place the animal on its back on a bench napkin and thoroughly douse the mouse and the surrounding area with 70% ethanol. This will not only sterilise the mouse but will also keep hair out of the incision. Make a key hole cut in the mouse by using forceps to pinch the mouse just below the sternum and cut through both the skin and fur. Take care not to cut through the membrane that holds the internal organs in place and what ever you do, do not cut into the intestines. They are full of bacteria that may contaminate your extracted cells and it smells bad!! Keep the scissors and forceps in a small beaker of 70% ethanol whenever they are not in use.

2. Using this cut as a starting point use surgical scissors to create a midline incision. Using fingers pull the sections of skin over the head and thighs taking care not to rip through the membrane.

3. Turn the animal, to expose the left side for easy access to the spleen. Make an incision into the left peritoneal wall with surgical scissors. The spleen is attached to the greater curvature of the stomach by pale pink connective tissue and is found behind the liver. The spleen generally has the same colour as the liver, but is a separate organ so be careful. The shape of the spleen (an elongated bean) is easily distinguishable from the liver.

4. Use the forceps to gently grab the spleen and use the scissors to severe all connective tissue. Place the spleen inside a Petri dish with 5mls of ice cold CCM and cover the Petri dish.

5. Examine the mouse further. Make sure you can identify the liver, the mesentery, the intestines with Peyer's patches. Can you find any lymph nodes?

6. Using the scissors and forceps, cut the spleen into 3-4 pieces.

7. Place the spleen fragments onto a strainer attached to a 50 ml tube. Press the spleen fragments through the strainer using the plunger end of a syringe.

8. Spin down the cells by centrifuging for 8 minutes at 300 rcf.

9. Once tubes have stopped spinning, carefully decant the supernatant into the waste liquid container. Resuspend the pellet in the remaining fluid by vigorously flicking the tube and then add 2 ml of the RBC lysis solution and flick to mix it with the suspended cells. Leave at room temperature for 5 minutes.

10. After incubation, add 12 ml of sterile PBS in order to stop the lysis.

11. Spin the cells down following the procedure above (step 11). Decant the supernatant and resuspend the pellet in 2mls of CCM.

12. Take 20µl of cell suspension to perform a cell count using trypan blue, as described in week-5 laboratory class.

13. Resuspend the cells at a concentration of 2 x 10^6cells/ml (live cell concentration) in CCM. Add 100µl of cell suspension to 15 wells of a 96 well plate. You will use less than half the plate and share the other half with other pairs of students.

14. Add 50µl of either the mitogens, antigen or medium to the cells in the plate, using the following plate template. You should record the COLUMN NUMBERS and ROW LETTERS that you use, on the plate template below.

	ConA	ConA	ConA			
	MCC	MCC	MCC			
	LPS	LPS	LPS			
	OVA	OVA	OVA			
	Medium	Medium	Medium			

15. Write your name, date and demonstrator on the lid of the plate and put into the 37^{o}C incubator with 5% CO_2. Also fill out the plate template form describing the contents of the wells, and write you name and demonstrator's name on the form and hand it to your demonstrator. The staff will need this if they are to confirm which sample is which for the next practical.

16. After 72 hours, supernatants will be harvested from the plates, pooled and frozen in order to later perform an IL-2 ELISA. This will allow you to test for activation of T cells.

CALCULATION OF CELL CONCENTRATIONS USED FOR YOUR EXPERIMENTS

WEEK 8 MEASUREMENT OF IL-2 USING AN ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

AIMS

- To demonstrate the activation of T cells in splenocyte cultures by the measurement of IL-2.
- To understand the basis for the differences in activation seen in cells stimulated by antigen or mitogen, and in cells from normal and transgenic animals.
- To understand the principles of ELISAs.
- To understand the concepts of assay specificity and sensitivity.

INTRODUCTION

This practical is a continuation of the practical from week six. You will receive supernatants from the wells of the culture plate that you set up. You will also be provided with samples that were prepared by students in the practical class held on the alternative day. The supernatants were extracted from the wells, combined and then spun to remove any cells or cellular debris. The cell free supernatants were then frozen until today's class. Today you will learn how to perform one of the most common immunological assays, the ELISA assay, which can be used to measure the concentration of almost any protein in solution. The ELISA assay is routinely used in research and diagnostic laboratories.

RISK ASSESSMENT

This class involves the use of supernatants from cultured suspensions of mouse leucocytes. While every effort is made to ensure that the supernatants are not infectious, this cannot be guaranteed. Thus, all procedures involving the handling of supernatants must be carried out using universal precautions. Gloves must be worn, and the use of safety glasses is strongly encouraged.

This class also involves the use of a microplate reader. Students must leave operation of the plate reader to their demonstrator or other qualified staff.

TMB (substrate solution) is a particularly hazardous toxin and should be handled with care. Wear all the proper safety equipment and blue nitrile gloves and goggles when using the substrate solution.

The stop solution is a hazardous corrosive. You must wear safety goggles in addition to the usual protective equipment when using this reagent.

DESIGN OF ASSAY TEMPLATE

Your demonstrator will discuss the use of standards to generate a standard curve. You must then design a template for your ELISA, keeping in mind the need for replicates, standards, blanks and dilutions.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

REAGENTS & EQUIPMENT

- Disposable gloves
- 1 ELISA plate pre- coated with anti-IL-2 capture antibody and blocked with skim milk powder
- 20 Eppendorf tubes
- 1 mL pipette and 100-200 µL pipette
- Blue and yellow pipette tips
- 2 mL recombinant mouse IL-2 (10 ng/ mL) in PBS
- 200 mL PBS-Tween in glass bottle (‘diluent’)
- 500 mL PBS-Tween in squirt bottle (‘wash solution’)
- Stock carboy of PBS-Tween
- 12 mL anti-IL-2 antibody conjugated to HRP
- 20 mL HRP substrate reagent
- 20 mL stop solution
- Paper towel
- Culture supernatants from previous week.

PROCEDURE

1. Make sure your template has been checked by your demonstrator before proceeding.

2. Label 10 x 1.5ml tubes per sample, and make 10 doubling dilutions of each of your recombinant mouse IL-2 standards using PBS as diluents. Add 300µl of the supernatant into tubes containing 300µl of PBS (see figure below). Make doubling dilutions of each of your supernatants in the same way. Use a clean pipette tip for each solution, but not for each dilution.

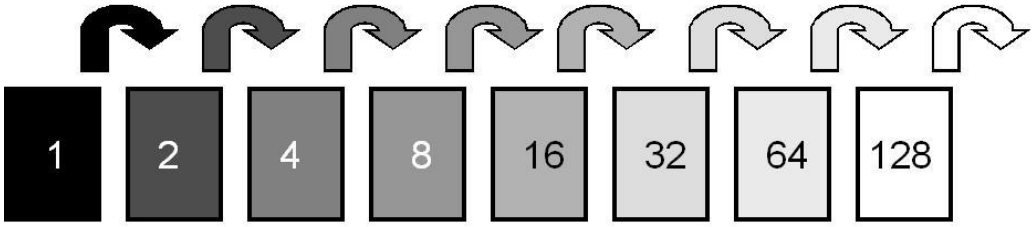


Diagram of doubling serial dilutions. Twice the required final volume of the sample or standard is added to well one. The required final volume of DILUENT is then added to wells 2-8. The required final volume is then removed from well 1, transferred to well 2 and mixed. Then the same volume is removed from the well and transferred to well 3 and mixed. This process is repeated across the plate, giving doubling dilutions out to 1/128.

3. The ELISA plates were coated with a monoclonal antibody to mouse IL-2 and were blocked already by the staff members from the preparation room. Add 100 µL of each dilution of each solution to duplicate wells as per your template.

4. Cover your plate with clingfilm and incubate at room temperature on the bench for 30 minutes.

5. Remove liquid from wells by 'flicking' the plate: ask your demonstrator to demonstrate. Blot the plate on a sheet of blotting paper to remove remaining liquid. Fill all wells with PBS-Tween using the squirt bottle. Flick off and blot plate.

6. Repeat step 6 twice more. This is known as 'washing' the plate.

7. Add 100µl of HRP-conjugated anti-IL-2 antibody to each well. Incubate on the bench for 30 mins.

8. Flick off liquid, blot, and wash twice with PBS-Tween as in step 6, blotting afterwards.

9. **WEAR GLOVES AND GOGGLES.** Add 100µl of substrate reagent to each well. Leave the plate to incubate on the bench until a blue colour

develops (approximately 10 minutes), then add 100µl of stop solution to each well.

10. Take your plate to be read on a plate reader. Make sure you take your plate template with you so the operator can correctly analyse your plate.

11. Record your results in the blank pages at the end of this practical. You need to record your standard curve and your raw absorbance data, as well as the calculated concentration of IL-2 in each of your test samples.

RESULTS & WORKING

RAW DATA REPORT

STANDARD CURVE REPORT

UNKNOWN CONCENTRATION REPORT

In the space below, record the concentrations of mitogen and antigen stimulated cells, as well as all control cells.

QUESTIONS

Why is the purpose of the ‘blank’ wells in an ELISA?

What is the purpose of the recombinant mouse IL-2 standard?

Why are replicates of all samples tested in an ELISA?

What are the differences between a ‘direct’ ELISA and an ‘indirect’ or ‘sandwich’ ELISA? Which type of assay are we using today?

What is IL-2 and what does it do? Why are we measuring it? Which cells produce it in the cell culture?

QUALITY CONTROL

Any assay that is used in either research or diagnostics must be carefully validated. Detailed studies must be undertaken to document parameters that describe the 'quality' of the assay. Two useful measures are the 'specificity' and 'sensitivity' of the assay. These issues will be considered during an incubation period later today.

We also want you to understand a number of other parameters that can be measured, which tell how well an assay has been performed. Although a series of tests may have shown an assay to have good specificity and sensitivity, we need to be able to check that YOU have performed the assay well.

> **Has the assay 'worked'? Will it provide us with <u>reasonable</u> measures of antibody concentrations in our samples? Is there some way we can decide this?**

Question: What possibilities might be open to us if we used the same standard and the same positive control sample in the assay every time it was performed?

The following ten estimates of the positive control concentration (in Relative Units / ml) have accumulated from ten plates assayed over a period of six months.

Results:

Positive Control (Undiluted)

1058, 1177, 1023, 945, 1119, 1083, 1222, 1131, 1036, 965

Your own assay gives you an estimate of the concentration of the positive control which is 875 Relative Units / ml.

Q1: Should you accept or reject the results of your assay? What are your reasons?

Q2. Using the accumulated results recorded above for the positive control sample, and with the help of the computer terminals in the lab, calculate the inter-assay coefficient of variation.

$$\text{coefficient of variation (CV\%)} = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

Calculations:

Positive control mean .....

Positive control S.D. .....

Assays having coefficients of variation that are less than 10% are usually considered to be good assays.

SENSITIVITY AND SPECIFICITY OF ELISA

The sensitivity of ELISA refers to its ability to correctly identify positive samples, and is therefore the proportion of the truly positive samples that are detected as positive. High sensitivity means a low number of false negatives.

$$\text{Sensitivity} = \frac{(true + ve)}{(true + ve) + (false - ve)} \times 100$$

The 'specificity' of an ELISA refers to the ability of the assay to correctly detect negative cases, and is therefore the proportion of the truly negative samples that are detected as negative. High specificity means a low number of false positives.

$$\text{Specificity} = \frac{(true - ve)}{(true - ve) + (false + ve)} \times 100$$

Consider the following table:

Sample	ELISA Outcome	
	Antibody +ve	Antibody -ve
Normal sera (population known not to have been infected by reference to an established bench-mark assay) (n=272)	2	270
Convalescent sera after proven infection (n=45)	40	5

How many false negative results were obtained? (How many genuinely infections were missed?)

What sensitivity does the assay therefore have?

How many false positives were seen?

What is the specificity of the assay?

WEEK 9 FLOW CYTOMETRY

AIMS

Today's practical class is related to the Diagnostic Immunology lecture, in which we talked about the principles of flow cytometric analysis and electrostatic sorting, as well as the use of flow cytometer in clinical laboratory. In addition to the clinical use, flow cytometer is also widely used by immunologists and other researchers as an important research tool.

At the end of this session:

1. You should be familiar with common cell markers that are used to identify different types of white blood cells in human peripheral blood.

2. You should be able to interpret flow cytometry data plots and to determine the identities of the major cell populations that are represented in the data plots.

3. You should be able to draw plots, including gating diagrams, of the kind that would be seen during routine immunophenotyping of human peripheral blood.

ACTIVITIES

1. Video tutorials
2. Complete the phenotypic marker table
3. Interpretation of clinical data
4. Visiting UNSW flow cytometry facility

INTRODUCTORY MATERIALS OF FLOW CYTOMETRY

Flow cytometers are machines that analyze particles flowing in a liquid stream.

Within the flow cytometer, cells (or particles) pass in single file through a laser light beam. Each passing cell generates light signals as they pass through the laser beam. These light signals are detected, converted into electric signals, and saved for later computer analysis. Data is collected from each passing cell, allowing careful characterization of each cell.

The signals measured are usually forward scatter (light scattered from the cells at an angle of 2° from the direction of the laser beam: proportional to cells size); side scatter (light deflected at right angles to the laser beam: dependent on cell contents and often said to measure 'granularity'); and fluorescence generated by the laser exciting fluorescent tags (fluorophores) on or in the cells.

Flow cytometry has the advantage over fluorescent microscopy that many cells can be analysed in a short time. As many as fifty thousand cells per second can be analysed. The primary laser is usually an argon ion laser that emits blue light of 488nm wavelength - ideal for exciting fluorescein (FITC), phycoerythrin (PE), and propidium iodide (PI), which are very commonly chosen markers. FITC emits green light, PE emits orange light and PI emits red light, when excited by the blue light of the argon laser. In this practical, you will also see the use of proprietary Alexa Fluor reagents (eg Alexa Flour 488) from the company Thermo Fisher Scientific. Other lasers are also usually available to excite additional fluorophors.

A new technology emerged in 2009 called Mass Cytometry or Cytometry by Time of Flight (CyTOF), which combines mass spectrometry with a flowing cell system. Rather than detecting fluorescent signals, cell markers are detected using antibodies that are labeled with pure metallic elements. This technique remains very expensive and is only used in research at the moment. Mass spectrometry avoids the problems that arise form spectral overlap of multiple fluorescing compounds, and so CyTOF is able to simultaneously analyse a much higher number of signals in a single run. Many labs have used 23 different labels in a single run, and it is hoped that one day as many as 100 different markers might be simultaneously analysed using this technique.

Flow cytometry can be used for many different types of analysis and some examples are shown below:

1. IMMUNOPHENOTYPING

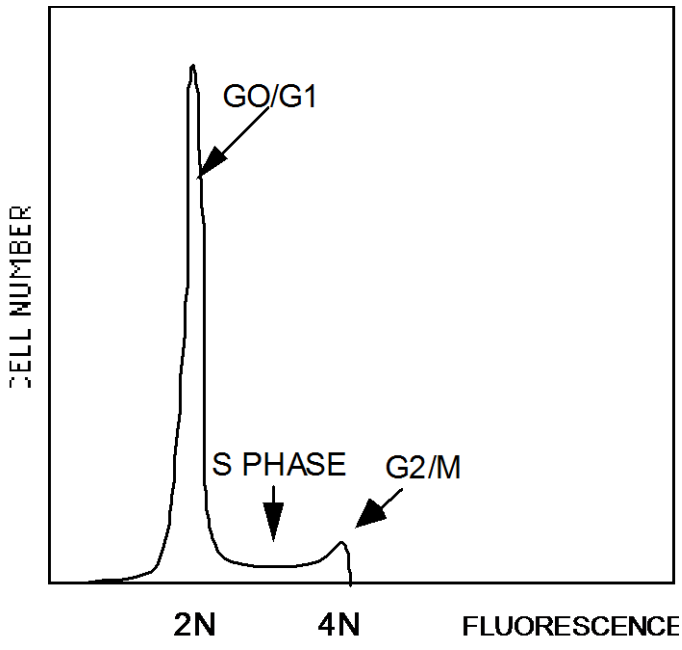
Immunophenotyping is a term for the phenotypic characterization of cells of the immune system. White blood cells are routinely immunophenotyped by flow cytometry. This involves the use of fluorophore-labelled monoclonal antibodies to define different cell populations, and to detect cell surface antigen expression by those cells. Cell samples are typically divided into aliquots, and pairs of monoclonal antibodies directed against different cell surface markers (with each antibody of the pair carrying a different fluorophore) are added to each tube. The cells are passed through the flow cytometer and fluorescence from each fluorophore is measured. The number of antibody molecules binding to cells varies enormously, so the fluorescence is typically expressed on a logarithmic scale. A recent variation of this is the use of the biexponential scale, described below.

By combining data from different cell aliquots, detailed information can be gained about the many cell types that may be present in the sample. This is the approach that is routinely followed to analyse blood cells in clinical immunology laboratories. With care, larger numbers of labeled antibodies can be added to each tube, and in research laboratories, by very carefully selecting fluorophores, it has become possible to simultaneously measure ten or more different cell surface markers.

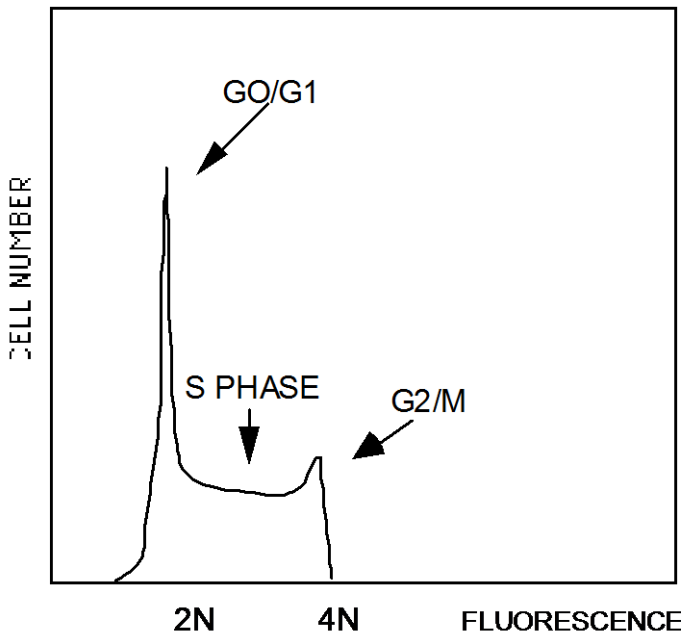
2. CELL CYCLE ANALYSIS, ALSO CALLED PLOIDY ANALYSIS

Propidium iodide is a fluorescent dye that binds stoichiometrically to DNA. That is, the amount of PI that will bind to a cell (and thus the fluorescence that the cell will emit) is proportional to the total DNA in the cell. PI can therefore be used to measure DNA levels in cell populations. When staining with PI, we include detergent to permeabilize the cells so that the PI can reach the nucleus. Normal human cells have 2 copies of each chromosome (**diploid** DNA) and all cells not dividing (G_o) or having just divided (G_1) will have the same amount of DNA. As cells prepare to divide, they synthesize DNA (S-phase), until just before mitosis (G_2) they have twice the diploid amount of DNA. Cells in G2 phase will therefore bind twice as much PI as cells in G1 phase and fluoresce with twice the intensity.

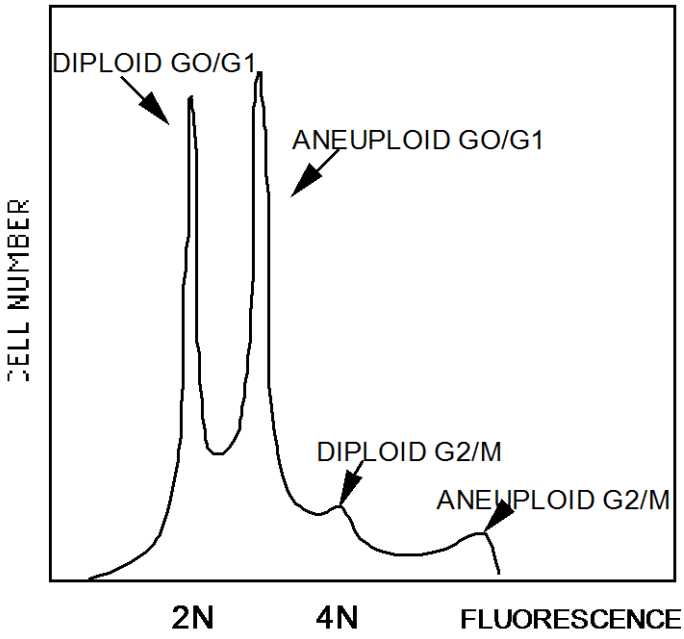
For normal cell populations, only a few percent of cells are dividing at any one time. All cells have normal numbers of chromosomes and the profile of PI fluorescence will look as shown in the following diagram.



For populations where all cells have normal numbers of chromosomes, but many cells are dividing, the PI profile will look like this:



In a cancer cell population, there may be more or less than the normal number of chromosomes, called an **aneuploid** amount of DNA. Cells with abnormal amounts of DNA will still cycle, so a mixture of diploid and aneuploid cells may look like this:



Thus staining with PI, it can tell us:

(1) Whether a cell population has normal numbers of chromosomes.
(2) What proportion of the cell population is actively dividing.

3. LIVE / DEAD CELL DISCRIMINATION

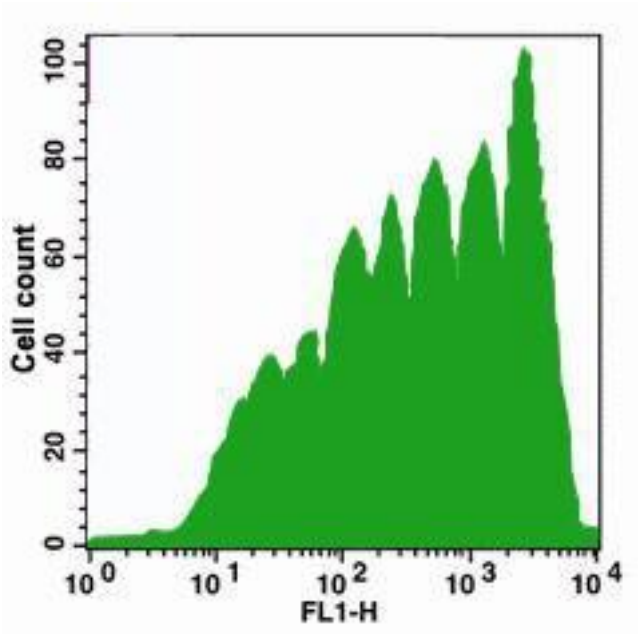
PI is used to study the cell cycle, but it can also be used to distinguish between living and dead cells. Although detergent must be used to permeabilise living cells, dead cells will be take up PI in the absence of permeabilisation. PI staining can therefore be used to identify LIVING and DEAD cells. Dead cells will stain with PI, while live cells will not.

4. IDENTIFYING TRANSFECTED CELLS

The gene encoding green fluorescent protein (GFP) is often used as a reporter gene when researchers try to introduce a gene of interest into mammalian cells. Expression of the gene of interest results in expression of the highly fluorescent GFP. Flow cytometry can therefore be used to distinguish between cells that have been successfully transfected, and cells that have not.

5. MONITORING CELL DIVISION

Fluorescent compounds which bind specifically and irreversibly to plasma membranes in a concentration dependent way can be used to detect cell division. As a stained cell divides and two daughter cells are formed, the fluorescence intensity on the cell surface is halved because it is shared between two cells. In the figure below, stained cells have been stimulated to divide. Undivided cells, with maximum fluorescence, are at the right-hand end.



VIDEO TUTORIALS

An excellent series of training videos are accessible at the following site.

https://www.thermofisher.com/au/en/home/support/tutorials.html

We will be watching two of the tutorials.

- Introduction to Flow Cytometry (12 minutes)
- Analyzing Flow Cytometry Data (17 minutes)

COMMON PHENOTYPIC MARKERS OF HUMAN PBMCS

Complete the table below (You can use internet searches)

	MAIN POPULATION	**OTHER POPULATIONS**
CD3		
CD4		
CD5		
CD8		
CD14		
CD16		
CD19		
CD20		
CD23		
CD27		
CD38		
CD45		
CD56		
CD138		

INTERPRETING CLINICAL DATA

A series of images of flow cytometry plots have been posted at the BABS3041 Moodle site. The plots were kindly provided by a Sydney clinical immunology lab.

Flow1

Flow1 shows the analysis of GATED LYMPHOCYTES from the lymph node of a normal healthy adult donor. The lymphocytes were gated using their forward scatter (FSC) and side scatter (SSC) characteristics.

Q1: In the top right plot, three populations have been coloured. B cells are blue, T cells are red and a third population is green. What kind of cells do you think are labeled green?

This manipulation of the plots is called 'color gating'. This means that the cells for example that are CD19+ CD3- are shown as blue events in all other plots of the data. It is therefore possible to follow the phenotypic characteristics of the B cells through the different plots, by focusing on the blue cells.

Q2: Note that in Flow 1, different antibody pairs have been used. Note also the different fluorescent labels that are used. Can you identify any antibodies in the plots that share the same fluorophore?

Two different antibodies, labeled with the same fluorophore, couldn't be used together to stain cells. The fact that some of the antibodies are labeled with the same fluorophore tells us that this data was collected by preparing cells, setting up a series of aliquots of the cells, then adding different pairs of labeled antibodies to each tube. We can build up a complex picture of our cell populations in this way, without dealing with the challenges of adding, say, 10 antibodies with 10 different labels to a single tube. Such challenging experiments with many different fluorophores are only seen in research laboratories. This is NOT a part of routine clinical investigations.

Q3: Can you explain why each antibody pair has been include in the analysis? (Ignore the CD10 and CD22 plot for now).

Q4: Can you see that kappa events are more common than lambda events? The kappa and lambda plots show that 10% of the lymphocytes of this individual are kappa positive, and 7% are lambda positive. This is a very typical ratio, with almost exactly 60% of the B cells being kappa and 40% lambda. There is variation in this value between individuals, but too much departure from the 60:40 ratio is a sign that something is not right.

Q5: What conditions can you think of that could lead to a change in the kappa and lambda ratio?

Flow2

Q: Flow 2 contains data from a patient. What is the outstanding feature of these plots? Can you think of a possible reason for the treatment this patient has received?

Flow3 and **Flow4**

Flow3 and **Flow4** are data from a single patient. **pblnad1** and **pblnad2** are data come from analysis of lymphocytes from a normal adult donor. (The lymphocytes have been gated on the basis of forward and side scatter. This plot is not included).

Q1: Compare the percentages of the different lymphocyte populations in the patient and the healthy control.

Q2. What condition do you think this patient might be suffering from? Record your reasons.

Q3. How do you explain the elevated and decreased percentages of various populations that you see in this patient?

Flow 5

Q1: Can you identify a population of cells that are present at an abnormal level in **Flow 5**?

Q2: What condition do you think the patient may be suffering from?

Flow6 and Flow7

These plots show immunophenotyping of bone marrow samples. First examine the Flow6 data carefully. These data come from a normal adult donor.

Q1: There is a large population of CD45 negative cells. What could these be?

Q2: Locate the plasma cell population. What are plasma cells doing in the bone marrow?

Now examine Flow7. What condition do you think this patient is suffering from?

DRAWING YOUR OWN DOT PLOTS

Before completing this practical class, you are required to demonstrate your understanding by drawing dot plots. Dot plots may not be the best way to present data, but they are the easiest way to do so, using a pen and paper.

1. First, draw a diagram of FSC-A vs SSC-A of normal blood sample

2. How can you further analyze B cell population?

WEEK 10 CLINICAL CASES

AIMS

- To apply the knowledge that you have acquired from the immunology course into real-life examples.
- To assist students to integrate different parts of the immune system into a coherent model.
- To introduce students to clinical immunology

INTRODUCTION

Today's practical class requires you to consider a number of clinical cases. You should determine possible immunological disorders that might be responsible for the problems seen in these cases. You are asked to consider appropriate immunological and other tests that you might order to aid your diagnosis. If the results of such tests are available, you are then required to interpret the results of those tests.

DIAGNOSTIC TESTS

The more commonly available assays for investigating the competence of the immune system include complete blood count, differential cell count, quantitative serum immunoglobulin levels, investigation of immunoglobulins by serum and urine electrophoresis, enumeration of blood T and B lymphocytes and major T cell subsets, complement function and phagocyte function. Together with a history and physical examination, these tests will identify the vast majority of patients with immunodeficiency.

Note that the results of some immunological tests may be reported as a percentage of the mean value seen in a normal population. That is, if the mean concentration of an analyte seen in a population was 40 units/ml, and 30 units/ml of the analyte was seen in a particular patient, this would be reported as 75% of the population mean.

Descriptions of the most commonly used techniques for assessing these parameters are presented below.

NEPHELOMETRY

This is a standard method for quantitation of the serum immunoglobulin classes IgG, IgA and IgM. It is also used for quantitation of a number of other abundant or moderately abundant serum proteins, such as the complement components C3 and C4. Specific antibody is added to a tube that contains the serum sample, and immune complexes then form. For example, if IgG is to be measured, anti-IgG

antibody is added to the tube. A light beam is passed through the tube, and immune complexes cause the beam to scatter. The amount of scattered light, generally detected at a 70˚ angle, is proportional to the concentration of immune complexes, and therefore the concentration of antigen (in this case, IgG) in the serum. These assays can be readily automated and several different antigens can be measured one after the other in the same serum sample. Note that the detection of a protein by antibody does not always mean that the protein is functionally active.

NEUTROPHIL FUNCTION

Several tests for neutrophil function are available. The most commonly employed are the NBT (nitro blue tetrazolium) dye reduction test, and chemiluminescence. These tests measure the oxidative burst that occurs on neutrophil activation, for example after contact with yeast or *Staphylococcus aureus* coated with antibody and complement. Normal neutrophils generate hydrogen peroxide and superoxide radicals, which are detected in the above tests.

COMPLEMENT FUNCTION

To screen for congenital deficiency of one of the components of the complement cascade, a functional assay of total haemolytic complement, such as CH_{50} or CH_{100} is performed. In these tests, the patient's serum is mixed with antibody-coated red cells. In normal serum, lysis of the red cells takes place. If any components of the classical pathway are deficient, the red cells will fail to lyse, and specific assays of the concentration of individual components are then performed using more expensive assay systems.

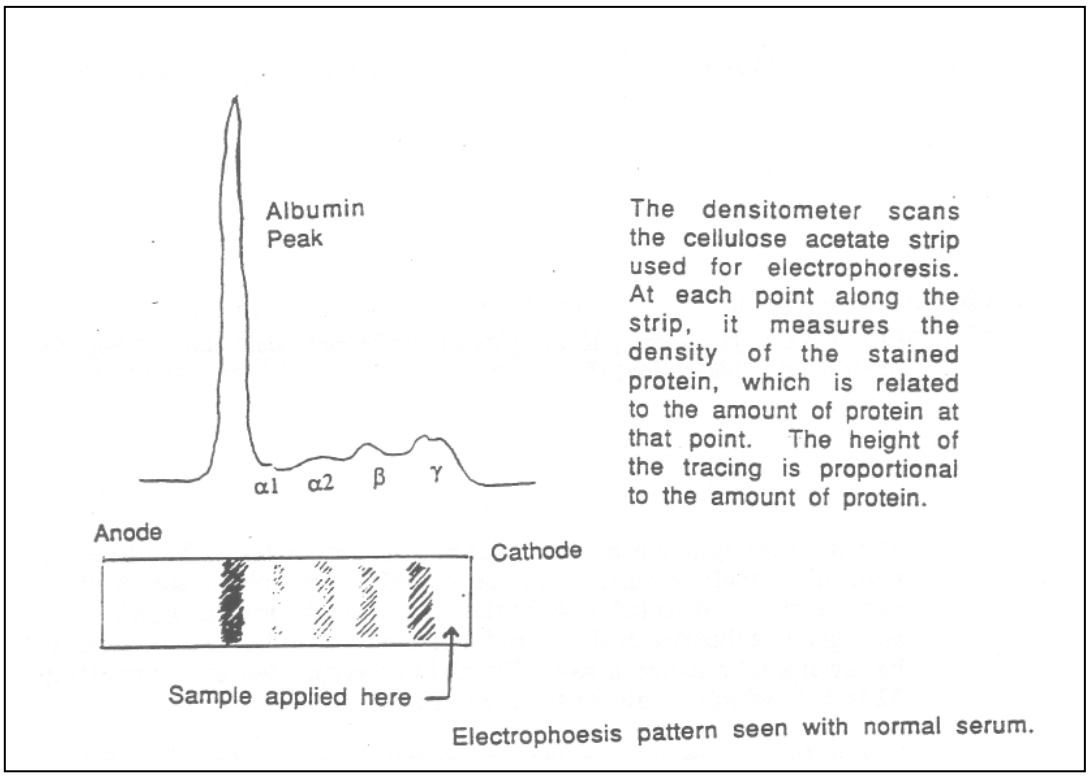
SERUM AND URINE ELECTROPHORESIS

These are most widely used to screen for the presence of monoclonal immunoglobulin, which may indicate malignancy of plasma cells or B cells. IgG deficiency is also readily apparent on serum electrophoresis. When serum is subjected to an electric field, proteins are separated according to their charge. Albumin is the main anodal band, followed by alpha 1-, alpha 2-, beta-, and gamma-globulins. The gamma globulin fraction is almost entirely composed of immunoglobulin, mainly IgG, whereas the alpha and beta regions comprise many different proteins. Some immunoglobulins, especially IgA, are present in the beta and even the alpha-2 regions.

The normal gamma region is a broad smear on serum electrophoresis, because the different amino acid sequences in the variable regions of immunoglobulins give rise to different surface charges, and therefore a range of electrophoretic mobilities. A malignant clone will secrete a large amount of immunoglobulin of the same amino acid sequence, and therefore the same electrophoretic mobility, so a narrow band will be present on the electrophoretogram. In such cases the urine often contains light chains, known as Bence-Jones protein, derived from the same malignant cells. Intact antibody is usually not found in the urine because it

is too large to be excreted by the kidney. In patients with multiple myeloma, when there is a large malignant clone, production of normal polyclonal immunoglobulin is usually suppressed, and there is increased risk of infection

After electrophoresis, gels are stained and scanned in a densitometer. Using values determined for total protein concentrations, and after integration of the densitometer scan, the concentrations of the various fractions may be estimated in g/L.



Electrophoesis pattern seen with normal serum.

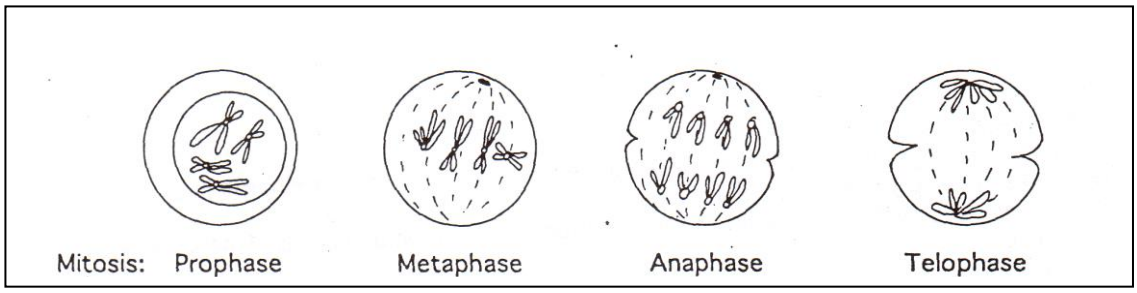
MITOGEN STIMULATION

A number of plant lectins are routinely employed to assess lymphocyte function. Phytohaemagglutinin (PHA), derived from kidney beans, and Concanavalin A (Con A), derived from jack beans, stimulate T cells to divide. Pokeweed mitogen (PWM) is a T cell-dependent B cell mitogen. Exposure of normal lymphocytes to PWM leads to the production of polyclonal antibodies.

ANTI-NUCLEAR ANTIBODIES (ANA'S)

The test is based on indirect immunofluorescence employing HEp2 cells, a human laryngeal carcinoma line. Cells are cultured on a microscope slide as a substrate. In a positive assay, the first step is the interaction of patient antibody with the nuclei of the cultured cells. FITC-labelled antibody to human immunoglobulins are then used to detect bound autoantibody. ANA staining patterns are then visualised using a fluorescence microscope.

Slides are evaluated under low power (10x) for presence of specific auto-antibody reactions. If positive, they are examined under high power (40x), for the identification of specific staining patterns. A positive reaction is the presence of any pattern of nuclear apple-green fluorescent staining. Serum is considered negative for ANA if nuclear staining is less than or equal to the negative control. When assessing mitotic figures, only cells in metaphase are examined.



By referring to the following descriptions of staining patterns, and to the charts on display, you should be able to identify some patterns.

You must ask.....

1. Are the nuclei staining?
2. What is the staining pattern?
3. Is there chromosomal staining?

STAINING PATTERNS:

Examples of the following staining patterns will be available. Before you consider case 8, you should examine the results of a series of anti-nuclear antibody tests that have been provided for us by the staff of St Vincent's Hospital.

Homogenous—solid diffuse staining of entire nucleus. Mitotic figures are positive. These antibodies are reactive with DNA-nucleoprotein-histone complexes. Seen most commonly in SLE and procainamide induced SLE. Also rheumatoid arthritis.

Speckled—Specks of staining dispersed throughout the nucleus. Mitotic figures do not stain. These antibodies are directed against nuclear antigens RNP, Sm, SSA (Ro) or SSB (La). Found in SLE, Sjögren's syndrome, polymyositis, scleroderma.

Nucleolar—Staining of the nucleolar membranes due to antibodies reactive with RNA-nucleoprotein complexes. Mitotic figures are negative. Found in Sjögren's syndrome, Raynaud's disease, myositis, scleroderma.

Centromere—Centromeres stain positively, as do the chromosomes in mitotic division - producing 46 dots per cell.

Peripheral—Similar to homogenous but the nuclear rim stains intensely and antibodies are directed against DNA.

Note that many other staining patterns are occasionally seen.

CASE STUDIES

CASE 1

The Van Doorns are an American couple who migrated to Australia. They are both descended from Dutch settlers who arrived in America in the seventeenth century. The Dutch settler community of their ancestors is known to have tolerated consanguineous marriages (marriage between close relatives), and Mr and Mrs Van Doorn are distantly related to one another. Their youngest daughter Petra, at age 10 months, developed a cold that persisted for a 14-day period. At this time, the child became drowsy and feverish. She was rushed to hospital after developing convulsive movements in her limbs, but died en route. Post-mortem cultures of blood, and cerebrospinal fluid led to the growth of the important bacterial pathogen, *Haemophilus influenzae*. At autopsy, Petra was found to lack a spleen - an extremely rare condition. Four siblings survive. While two of the children (Karel and Sonja) are well, the other two (Jan and Lies) have histories of repeated serious infections including otitis media, pneumonia and meningitis. *Haemophilus influenzae* type b and another encapsulated organism, *Streptococcus pneumoniae*, have been responsible for these infections.

All the Van Doorn siblings have been immunised with the DPT vaccine for protection against diphtheria, pertussis (whooping cough) and tetanus. An assay was conducted to measure the presence of vaccine-induced antibodies in their serum. A haemagglutination assay was used in which red cells are coated with a test antigen, and then incubated in the presence of dilutions of serum.

Examine the haemagglutination plate provided and record the titre of the serum of each child. Make sure you understand why the controls appear as they do.

Q1: Do you think there is a significant difference between the responses of the healthy and sick children?

The immune function of the children was further tested by challenging them with additional vaccines. All the children were challenged with a typhoid vaccine that is directed against the pathogen *Salmonella typhi*. The vaccine was given subcutaneously. Production of antibodies was measured using a bacterial agglutination assay. The children responded with titres ranging from 32 to 128, which are all considered positive.

The children were then challenged intravenously with 1ml of a 25% suspension of sheep red blood cells.

Q2. How could you very easily measure the response of the children to this innocuous antigen?

Ask your demonstrator for the results of the test, and record your observations.

Imaging technology was then used to try to visualise the spleens of these children. Ask your demonstrator for the results of this examination.

Q3. In light of these images, why do you think some of the children had a normal response to the typhoid vaccine, but not to the sheep red blood cells?

Q4. Time passes, and the Van Doorns have a further three children. While the parents are normal, three of the eight children are born without spleens. One of these asplenic children grows up and has children of her own - four boys and a girl. They are all normal. What is the inheritance pattern of congenital asplenia in this family? According to Mendelian laws, how many of the original eight Van Doorn children would be expected to have no spleen?

Q5. Asplenia is VERY RARELY congenital, but quite often the spleen is removed after trauma. What special precautions would you advise for a patient who had their spleen removed after it was ruptured in a serious car accident?

CASE 2

A male infant, three months of age, is brought to your surgery. You have seen him on a number of occasions recently, for he has suffered repeated pyogenic infections (otitis media, pharyngitis, dermatitis etc). The causative organisms have been Staphylococcus aureus and Streptococcus pyogenes. These are well-recognised bacterial pathogens. No obvious history of viral or fungal infections and no lymphadenopathy can be felt on palpitation. The boy received DPT vaccination at 2 month of age. A 3 year old female sibling is unaffected by such problems.

Q1. You are concerned that your patient may be suffering from a congenital immunodeficiency. Considering the kinds of infections this patient is reported to have suffered from, which of his immunological compartments may be deficient?

The following tests were performed:

. whole blood count and lymphocyte subsets

. lymphocyte phytohaemagglutinin stimulation

. neutrophil chemiluminescence

. nephelometry quantitation of immunoglobulin concentrations

. CH100

. diphtheria and tetanus antibody test

Ask your demonstrator for the results of the tests.

Q2. What is your interpretation of these findings?

Q3. What circumstances, other than a primary immunodeficiency, could account for the failure of a child to respond to vaccination?

After treatment, lymphocyte subsets were again measured and immunoglobulin concentrations determined.

Ask your demonstrator for these results

Q4. In the light of this information, how do you think the patient might have been treated?

Genetic testing was performed to investigate possible causes of this child's condition, and a mutation of the *btk* gene was identified. **Why do you think a defect in *btk* leads to the kind of disease you see presented here?**

CASE 3

A four month old child presents with multiple recurrent infections including CMV and other viruses, pneumonia (both bacterial and Pneumocystis carinii), oral thrush - a fungal infection, and a protozoan infection.

Q1. What immunological functions, or compartments, are likely to be deficient in this child?

The following tests were performed on this patient:

- whole blood count and lymphocyte subsets measurement
- lymphocyte function tests
- nephelometry quantitation of immunoglobulin concentration
- chest x-ray

Ask your demonstrator for the laboratory reports

Q2. What do these test results suggest about the patient's immunological deficit?

Q3. What special hazards would sometimes accompany vaccination of these patients?

Genetic testing was performed to investigate possible causes of this child's condition, and a mutation of the gene that encodes the common gamma chain of the IL-2R was identified. **Note the multiple effects that a loss of function of this chain can have. Not only does it compromise signalling by IL-2, but many other cytokines are also unable to function**.

CASE 4

A 17 month old boy with a skin abscess, persistent lymphadenitis and hepatosplenomegaly is seen. Staphylococcus epidermidis, a normally innocuous member of the skin microbiota, is abundantly isolated from the abscess pus. The child has a long history of pyogenic (pus-forming) mucosal and systemic infections, often involving organisms of low virulence. These infections have included 1 bout of osteomyelitis, pneumonia, sinusitis and dermatitis.

Q1. What immunological abnormality may be present?

Q2. What tests of immune function would be appropriate?

Ask your demonstrator for the results of the tests you have selected

Q3. What do the reports indicate? What does the mother's report tell you about the condition from which the boy suffers?

Q4. What treatment of the condition is available?

Genetic testing was performed to investigate possible causes of this child's condition, and an X-linked mutation of one of four genes that encode the subunits of phagocyte NADPH oxidase was identified. This enzyme generates microbicidal oxygen radicals during the 'respiratory burst'.

CASE 5

Miles Chapwood led a normal childhood until around the age of twelve when he began to lose weight and to develop an enormous thirst. His excessive fluid consumption was matched by frequent urination. His family doctor promptly diagnosed juvenile onset diabetes mellitus - an autoimmune disease in which the immune system attacks the islets of Langerhans. Miles was started on daily insulin injections, and responded well. He remained fit and active throughout his childhood and early adulthood. However at the age of 35 years, his physician noted an elevated blood pressure. A serum creatinine test was performed and levels were markedly raised. This led the physician to suspect renal complications of diabetes. The diagnosis was confirmed after kidney biopsy, which suggested Miles was in imminent danger of kidney failure. He began twice-weekly haemodialysis and was put on a waiting list for a cadaveric kidney transplant.

Miles was blood group B, Rh-positive. His HLA-type was A2, 24; B50, 51; DR3,4. After a long wait, a cadaveric kidney became available. The donor was blood type B, Rh-positive and his HLA type was A2, 11; B7, 35. No information on the DR type was available. Miles blood was tested for antibodies directed against the white cells of the donor, and was found to be negative.

The transplantation was made, and Miles commenced treatment with drugs including the immunosuppressive drug cyclosporin. One week after his discharge from hospital, signs of rejection appeared. Additional immunosuppressive drugs were prescribed, as well as anti-CD3 antibodies. Six weeks later, Miles developed a fungal infection of the lungs. This was successfully treated with anti-fungal drugs. He has remained in good health since then, and continues on his immunosuppressive therapy.

Q1. Why was it considered more important to match the potential donor's blood group with Miles' blood and to ascertain that Miles had no cytotoxic antibodies to the donor than to worry about complete HLA matching?

Q3. Why do you think Miles was treated with anti-CD3 antibodies?

CASE 6

A 37 year old male patient presented with a recent history of shortness of breath and dry cough. He reported night sweats and fevers. He complained of progressive weight loss and lethargy over the last three months. Chest X-ray demonstrated bilateral interstitial infiltrates with patchy areas of consolidation. In addition to conducting microbiological tests, flow cytometric analysis of peripheral blood cells was performed.

Consider the results of the flow cytometric analysis of the patient's mononuclear cells.

Q1. What % of the lymphocytes are T cells?

Q2. What types of cells are the CD3-negative lymphocytes?

Q3. The sum of the CD4 and CD8 results does not exactly equal the CD3 result. Why might this be so?

Q4. What is the patient's likely underlying disorder? What tests could you order to confirm this diagnosis?

Q5. Which conditions, other than infectious diseases, is this person at increased risk of developing?

CASE 7

A 68 year old female with a recent history of pneumonia, urinary tract infection and radiating back pain has been hospitalised as a result of a spontaneous fracture of the femur. She is weak and slightly anaemic.

1. Can you think of any connection between the long bones and the immune system? Could there be an immunological basis for the skeletal problems of this patient?

The following tests were performed:

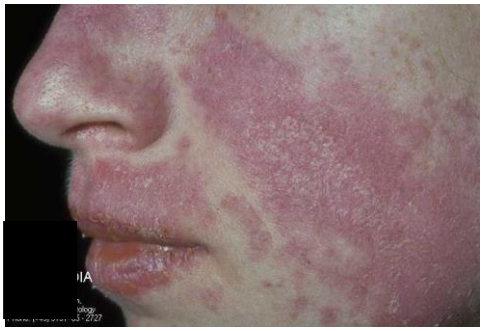
- chest, skull and long bone x-ray
- whole blood count
- serum electrophoresis
- nephelometric quantitation of immunoglobulin concentrations
- bone marrow biopsy, subsequent to x-ray findings.

Ask your demonstrator for the laboratory reports

Q2. What is your interpretation of these findings?

CASE 8

Neralie Childrop spent a happy day at Bondi beach, basking in the summer sun. She certainly overdid the tanning, and paid the price, with rather painful sunburn. Several days later, the sunburn was getting better, but a mask-like rash then appeared on her face (see picture below). Upon questioning, the patient reported that for some time she had experienced joint pains in her fingers and hips.



A test for anti-nuclear antibodies was ordered, and the results may be seen on the slide that is available for your inspection. Is the patient ANA positive, and if so, what is the staining pattern that you see?

Further tests were ordered, and it was shown that the patient's C3 levels were abnormally low.

Q1. Why might a condition involving high levels of production of anti-nuclear antibodies be associated with low levels of complement?

Q2. Tests were ordered of kidney function. Why might this condition be associated with altered kidney function?

Q3. What kind of therapy do you think could be useful for such a condition?

Note: The basis for the association between sun exposure and the development of symptoms in this condition remains unclear.

HLA TYPING

A haplotype is the combination of alleles at a number of loci on a single chromosome. Recombination in the HLA region of the chromosome is rare, being seen in only 1% of individuals examined. In the absence of recombination, an HLA haplotype is inherited from each parent. With recombination, a 'new' haplotype is created.

Q1: HLA typing of a couple showed them to carry the following HLA alleles (ignoring other HLA loci):

A1 B27 DR3 (on one chromosome)

Father:

A2 B7 DR7 (on the other chromosome)

A26 Bw44 DR2

Mother:

A11 B5 DR10

Assuming there is no recombination, list the possible HLA antigens expressed by their children.

Donors for kidney transplants are chosen after testing for mismatches at the A, B and DR loci. It is best to find a perfect match, but if this is not possible, mismatches should be kept to a minimum. Experience has shown that DR mismatches lead to less graft survival than mismatches at the A and B loci.

Q2: Why are rejection episodes experienced at all in HLA-matched transplants?

A group of siblings was found to have the following HLA antigens:

	A locus	B Locus	DR Locus
Marvin	A1 Aw68	B7 B12	DR2 DR3
Leota	A1 A9	B8 B14	DR3 DRw10
Kirsten	A1 Aw68	B7 B12	DR3 DR3
George	A1 Aw68	B8 B12	DR3 DR3
Roland	A1 Aw68	B8 B12	DR3 DRw10
Hilda	A1 A9	B7 B14	DR2 DRw10

Note: These alleles are listed in 'numeric' order. The table is not meant to indicate, for example, that Marvin has inherited the haplotype A1 B7 and DR2. A1 may be part of a haplotype that includes B12 and/or DR3.

Q3. Has there been recombination in this family? What is the evidence?

The family has been devastated by acute post-streptococcal glomerulonephritis. Perhaps this has something to do with the reported association between this condition and the B12 allele. We will never know. At any rate, serious damage has been done to Roland's kidneys and he requires a transplant.

Q4. Which sibling do you think would be the best donor?

> (What follows would not be considered ethical, but for the sake of the story….) Roland is told by his doctor who the best sibling donor would be. Roland wants to ask his sibling for this 'gift of life'. Roland begs his sibling for a kidney, saying, 'If you were the one needing the kidney, I would gladly give you mine.' But would Roland be the best choice for that sibling?

Q5. If this sibling really needed a kidney, who would be the best source of the kidney?

Q6: Misfortune continues to plague the family. Now Marvin needs a transplant. Which sibling should be asked to donate?