Analysis of Immunotoxicity by Enumeration of Antibody-Producing B Cells

Immunotoxicology is the study of adverse effects on the immune system resulting from occupational, environmental, inadvertent, or therapeutic exposure to chemical or biologic materials. The immune system is a target for chemical-mediated damage, and up- or down-regulation of its activities can result in significant health problems. Because of the increasing number of identified immunosuppressive diseases, it has become even more important to determine the potential effects of xenobiotics on the immune system.

Many immune-associated diseases are due to an altered function of the acquired immune response. The acquired immune response can be divided into two major classifications—humoral immunity (HI) and cell-mediated immunity (CMI), which reflect the primary effector functions of B and T lymphocytes, respectively (Janeway, 1999). While these responses are not mutually exclusive, they provide distinctly different avenues for dealing with pathogenic organisms or altered host cells. CMI is primarily involved in the defense against intracellular pathogens and altered host cells, while HI is primarily involved in the defense against soluble antigens. This unit describes methods to evaluate the effects of immunotoxic xenobiotics on the humoral immune response.

Humoral immunity is mediated by antigen-specific antibody–producing B cells. Secreted antibodies bind to antigens on the surfaces of invading microbes, and this flags them for destruction. In the immune response, IgM is the first immunoglobulin produced in response to antigen stimulation. Cells that produce IgM, or their precursors, do not become memory cells; a second exposure to antigen initiates a class switch that results in the production of IgG antibodies instead of IgM and is necessary for the formation of memory cells. The heightened response to secondary exposure to the antigen is due to affinity maturation of antigen-specific clones and the activation of memory B cells (Heyman, 2001). Regulation of the humoral immune response is complex and depends on several factors including: type of antigen, amount and route of exposure, cell types participating in the response, and the genetic capabilities of the cells. Humoral immunity is often analyzed in a laboratory using characterized antigens in either an in vivo or in vitro system. Sheep red blood cells (sRBC), keyhole limpet hemocyanin (KLH), and pigeon cytochrome *c* are T-dependent antigens for which an optimum antibody response requires T lymphocyte assistance (Lane, 1995). There are also a small number of antigens capable of activating B cells with only minor help from T cells; these are called T-independent antigens (Mond et al., 1982). In general, primary antibody responses (IgM) to T-independent antigen analyzed in vitro are weaker and peak earlier than the responses to T-dependent antigens. The secondary response to a T-independent antigen in vitro also differs from a T-dependent antigen in that it too is weak and consists only of an IgM response. Therefore, these antigens do not induce maturation of the humoral response or class switch to IgG, IgA, or IgE.

Due to the complexity of the immune system, assessment of chemical-induced immunotoxicity in laboratory animals has historically been performed through a tiered approach using multiple assays (Luster et al., 1988). The first tier provides a concise collection of tests to screen chemicals for potential toxicity on the immune system, including the antibody plaque-forming cell (PFC) assay or the IgM response to sheep red blood cells (sRBC). It has been suggested that this response is the most commonly affected functional parameter in animals exposed to chemical immunosuppressants (Descotes, 2004).

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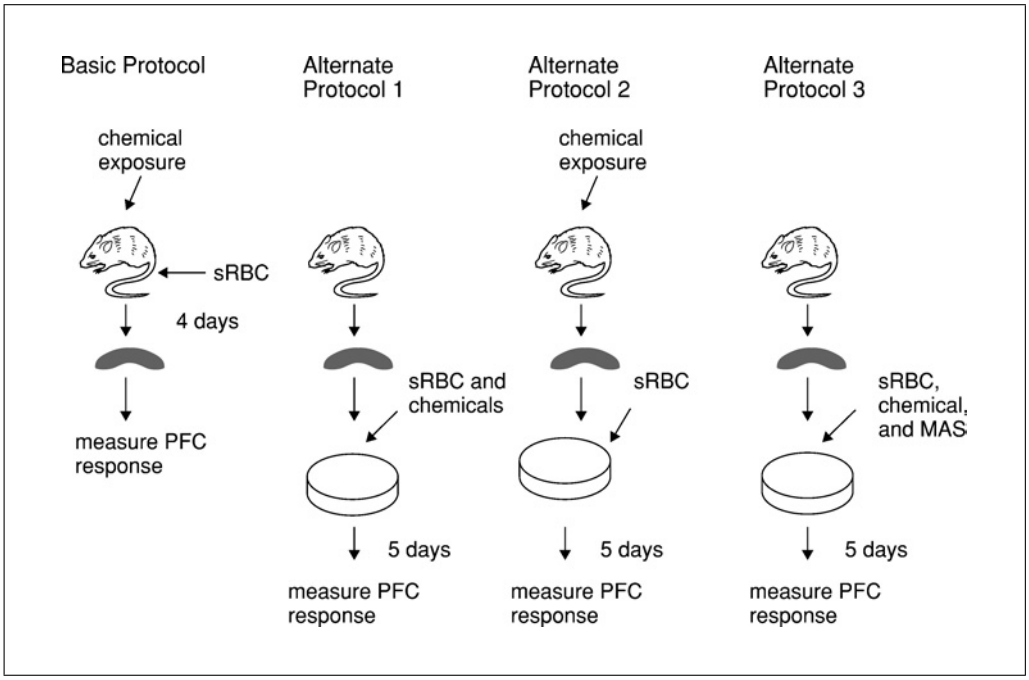

Figure 18.11.1 Protocol variations for analysis of antibody forming B-cells. Abbreviations: MAS, metabolic activating system; sRBC, sheep red blood cells. Adapted from Holsapple (1995).

Studies have shown that the PFC response to sRBC is not only an excellent monitor of the primary effector function of the B lymphocyte (i.e., synthesis and secretion of antigen-specific antibody), but it may also be the most sensitive immune parameter currently available to identify chemical perturbation.

Although a variety of methods are available to measure antibody-presenting B cells, IgM PFC response to sRBC in vivo (Basic Protocol) is a commonly used and widely accepted technique and will therefore be the main focus of this unit. There are several variations of this assay, illustrated in Figure 18.11.1, which will also be described: Alternate Protocol 1 describes an in vitro PFC assay for cell response to sRBC (Mishell-Dutton assay); Alternate Protocol 2 measures the effect of in vivo chemical exposure on in vitro PFC response to sRBC; and Alternate Protocol 3 measures in vitro PFC response to sRBC in the presence of a metabolic activation system, thereby allowing the immunotoxic effects of the metabolites of a test chemical to be studied. Finally, Alternate Protocol 4 describes an in vitro assay for cell response to T-independent antigens.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

BASIC PROTOCOL

THE PLAQUE-FORMING CELL (PFC) RESPONSE TO SHEEP RED BLOOD CELLS IN VIVO

The PFC assay measures the humoral immune response mediated by the combined actions of an antigen-presenting cell (usually the macrophage), T lymphocytes (required for the production and release of lymphokines and cell-to-cell contact), and B lymphocytes. The most common form of the plaque method is used for the detection of murine primary IgM antibodies directed against the T cell–dependent sheep red blood cell (sRBC) surface antigens.

Following exposure of animals to an antigen, antibody-producing cells (B lymphocytes) can be recovered from the spleens and enumerated by counting plaques, which are zones of hemolysis in a background of intact red blood cells (Jerne and Nordin, 1963). At the center of each plaque is the mature plasma cell. Hemolysis results from interaction of complement with the antigen-antibody complex on the surface of the red cell. The number of plaques formed is equal to the number of plaque-forming cells (PFC) present in culture and represents a measurement of the humoral limb of the immune system. Chemically induced changes in T cells, macrophages, or other antigen-presenting cells, or in B cells, can result in a decrease in PFC activity.

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

Materials

Male and/or female mice: typically B6C3F1 female mice (Taconic Farms) are used for this assay (Koller and Exon, 1985)
Potentially immunotoxic chemical for testing along with vehicle control and positive control (typically, cyclophosphamide)
HBSS/HEPES (see recipe)
3.75×10^8 cell/ml suspension of sheep red blood cells (sRBC) in HBSS, pH 7.0 (Support Protocol 1)
Bacto Agar (BD Biosciences)
30 mg/ml DEAE-dextran (prepared from dextran of average mol. wt. of 500,000; Sigma) in 0.9% (w/v) NaCl (adjust pH to 6.9 when dissolved; store up to 4 months at 4°C)
70% ethanol
Guinea pig complement (GPC), lyophilized (Cedarlane Laboratories; *http://www.cedarlanelabs.com*)
50% (v/v) suspension of sRBC (unprocessed, from same sheep donor/blood stock as sRBC used for immunization) in Alsever's solution (see recipe for Alsever's solution)
Hanks' balanced salt solution (HBSS), pH 7.0 (see recipe)
Isoton (Beckman Counter)
Zap-O-Globin II (Beckman Coulter)

12 × 75–mm disposable borosilicate snap-cap glass tubes and corresponding racks
1-ml syringes with 25-G 5/8-in. needles
100 × 15–mm polystyrene petri dishes
250-ml Pyrex Erlenmeyer flask
43° to 45°C water bath
Surgical instruments: scissors and forceps
5-ml plastic capped tubes
Balance (accurate to ± 0.5 mg)
60 × 15–mm petri dishes
Frosted microscope slides
Refrigerated centrifuge
15-ml conical centrifuge tubes
45 × 50–mm cover slips (each weighing ~750 mg; Fisher)
36° to 38°C incubator with thermometer
Plaque viewer (Bellco Biotechnology) or inverted microscope
Accuvette II vials (Beckman Coulter)
Coulter Counter (Beckman Coulter)

Additional reagents and equipment for intravenous (i.v.) injection of the mouse (Donovan and Brown, 2006a), euthanasia of the mouse by CO_2 asphyxiation (Donovan and Brown, 2006b), and counting cells (*APPENDIX 3B*)

Prepare for assay: Treat mice with chemical of interest and prepare tubes/petri dishes

1. Expose mice to the potentially immunotoxic chemical of interest using appropriate methods.

   The specific study design and dosing procedure will depend upon the type of chemical being investigated, and is at the discretion of the researcher. Each study should contain a vehicle control, dose response for the chemical, and a positive control. Cyclophosphamide (25 mg/kg body weight intraperitoneally in a 200-μl volume for the 4 consecutive days after immunization), a well characterized potent immunosuppressant, is commonly used as a positive control for this assay (Koller and Exon, 1985). A power analysis can be performed to determine the exact number of mice required for optimal study design when increased variability is expected. However, five mice are typically sufficient for each group, yielding 25 to 30 mice per study.

2. For each mouse, label one 12 × 75–mm snap-cap tube with the mouse ID number and add 3 ml of HBSS/HEPES to each. Also for each mouse label one 100 × 15–mm petri dish with the mouse ID number and the dilution of the spleen cell suspension that will be used for plaquing on day 4 (1:30 in the plate top and 1:120 in the plate bottom).

Immunize mice 4 days prior to plaque assay

3. Immunize each mouse by injecting intravenously (Donovan and Brown, 2006a) with 0.2 ml of a 3.75×10^8 cell/ml suspension of sRBC (7.5×10^7 sRBC total) using a 1-ml syringe and 25-G, 5/8-in. needle.

   The sRBC used in this immunization step are prepared as in Support Protocol 1.

Day 4: Prepare agar

4. Prepare a working agar solution containing 0.5% (w/v) Bacto Agar and 0.05% (w/v) DEAE-dextran in HBSS/HEPES on the morning of the assay, as follows. Add the agar to the HBSS/HEPES at 0.5 g per 100 ml in a 250-ml Erlenmeyer flask and dissolve while heating to a boil (do not reboil). Next, add 1.6 ml of 30 mg/ml DEAE-dextran per 100 ml. Mix by gently swirling.

5. Dispense 0.50-ml aliquots of the warm agar into 12 × 75–mm disposable tubes which are held in a 43° to 45°C water bath, setting up two tubes for each animal used in the study (for two different dilutions of cells) and several more to test for blood contrast (see steps 17 to 18, below). Wait at least 15 min before plaquing so that the temperature of the agar can equilibrate with that of the water bath.

Day 4: Dissect spleens from sRBC-immunized mice

6. Weigh mouse to an accuracy of one decimal place (e.g., "23.5 g") and then euthanize with CO_2 (Donovan and Brown, 2006b). Spray the mouse and surgical instruments with 70% ethanol prior to dissection to prevent contamination.

   The weights of the mice should be taken at the beginning and end of the experiment. A decrease in weight could indicate toxicity of the chemical.

7. Use one pair of forceps and scissors to cut the skin and expose the underlying musculature. Open the body cavity and aseptically remove the spleen with a second pair of scissors and forceps.

Prepare spleen cell suspension

8. Tare a 5-ml plastic capped tube containing 3 ml of sterile HBSS/HEPES to an accuracy of the third decimal place, then place the spleen in the 5-ml tube, place the tube back on the balance, and record the weight to an accuracy of the third decimal place (e.g., "0.089 g"). Keep spleens on ice at all times.

   The spleen suspension should be prepared as soon as possible.

Table 18.11.1 Preparation of Dilutions for the Assay

Dilution	Cells	HBSS/HEPES (ml)
1:30	0.1 ml original stock	2.9
1:120	1 ml 1:30 dilution	3.0

The immunized spleen will be larger in size than an unimmunized spleen. Immunosuppressive chemicals will often cause a significant reduction in spleen weight and cell count.

9. Pour the spleen and 3 ml of HBSS/HEPES into a 60 × 15–mm petri dish.
10. Place the spleen between the frosted ends of two microscope slides. Move the frosted ends of the slides up and down several times to create a single-cell suspension of the splenocytes.

    This procedure has been shown to generate a single-cell suspension. Successful single-cell dispersion can be confirmed by use of a hemacytometer (APPENDIX 3B).

11. Use a Pasteur pipet to transfer the entire suspension back into the original 5-ml plastic capped test tube. Leave the connective tissue behind. Rinse the slides and petri dish with the medium to ensure the highest recovery of cells.
12. Centrifuge the cell suspension 10 min at 300 × *g*, 4°C. Resuspend pellet in 3 ml of cold HBSS/HEPES and place on ice for use in the assay and for determination of cell number as described in steps 25 to 27.
13. Prepare two dilutions of spleen cells (1:30 and 1:120) in cold HBSS/HEPES and hold on ice until they are needed for plaquing (see Table 18.11.1).

Prepare sRBC and guinea pig complement (GPC) for plaquing

14. Prepare sRBC for plaquing by adding 5 ml of a 50% suspension of sRBC in Alsever's solution to a 15-ml conical tube containing 10 ml HBSS/HEPES. Centrifuge 10 min at 1050 × *g*, 4°C. Remove the Alsever's/HBSS/HEPES supernatant and wash the sRBC twice more, each time by adding 10 ml HBSS/HEPES, centrifuging again as before, and removing the supernatant.

    IMPORTANT NOTE: *The sRBC used for plaquing are collected in Alsever's solution from the original sheep donor and are not processed as in Support Protocol 1. This is in contrast to the sRBC used for immunization (see step 3). The same blood stock should be used for immunization and plaquing, however.*

15. Suspend the final sRBC pellet in an equal volume of HBSS/HEPES for a final concentration of ∼1.2 × 10^{10} cells/ml.
16. Resuspend lyophilized guinea pig complement (GPC) according to the manufacturers' instructions. Dilute 1 part resuspended GPC with 3 parts HBSS before plaquing.

    The complement is temperature sensitive and needs to be kept on ice at all times.

Set up plaque assay

17. Add 20 to 30 μl of the sRBC suspension prepared in step 15 to a test tube containing 500 μl of the agar mixture (see step 5). Remove the test tube from the water bath and vortex briefly. Pour the entire sample into a 100 × 15–mm petri dish.
18. Breathe on a 45 × 50–mm cover slip to moisten it slightly and drop on the sRBC-agar aliquot. Allow the agar to set for 5 min and then examine for color contrast.

    In the PFC assay described in the subsequent steps, plaques form due to lysis of the sRBC. This results in a clear spot contained within a background of agar (Fig. 18.11.2).

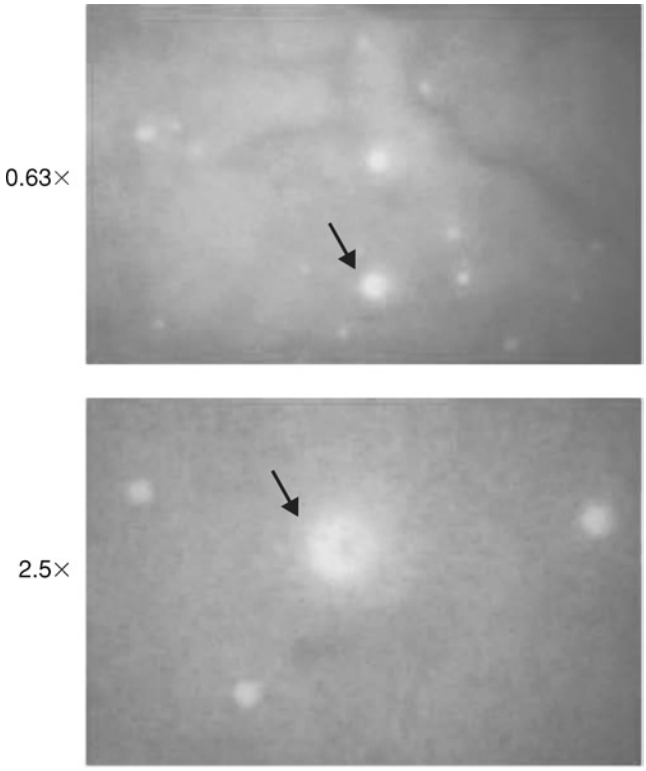

Figure 18.11.2 Light micrograph of plaques formed by hemolysis at 0.63× and 2.5× magnification. For the color version of this figure go to *http://www.current protocols.com.*

A background color dark enough to allow plaques to be seen but not dark enough to obscure them is ideal. If the contrast is too dark, fewer sRBC should be added and if too light then additional sRBC should be added. The volume of sRBC determined in this way gives the best color contrast for plaquing the cultured splenocytes.

19. Add the optimal volume of sRBC to each of the agar tubes set up for the samples in step 5 (this can be done for 16 to 32 tubes at a time).

20. Add 0.1 ml of the test cells (1:30 or 1:120 dilution; see step 13) to one tube at a time.

21. After the test cells are added, remove the tube from the 43° to 45°C bath, quickly add 25 µl of the reconstituted GPC (from step 16), gently vortex the tube, and pour into the corresponding top or bottom of the 100-mm petri plate labeled in step 2. Before the agar has solidified, breathe on a 45 × 50–mm coverslip (weighing ∼750 mg) to moisten it slightly and drop on the sRBC-agar aliquot.

22. When the agar is solid, put the plates back together with the top of the plate on the bottom part as in the usual plate setup and incubate at 36° to 38°C for 3 hr in a standard incubator (humidification not needed).

Count plaques

23. Using a plaque viewer or an inverted microscope, count the plaques on either the 1:30 or 1:120 dilution plate, depending on which dilution yields ∼100 to 300 plaques. Disregard plates with fewer than 100 or more than 300 plaques to ensure accurate counting.

Each clear circular spot in the agar (hemolysis of sRBC) represents one antibody-producing B cell and is counted as one plaque (Fig. 18.11.2).

24. Store the plates overnight at 4°C in case additional counts are required.

Determine number of splenocytes

25. To identify the number of splenocytes in the samples, add 20 µl of the original spleen cell suspension from step 12 to 10 ml Isoton in an Accuvette II vial.

26. Add two drops of Zap-O-Globin II just prior to Coulter counting.

27. Set the Coulter Counter to a diameter range between 3.3 and 6.647 µm and count cells.

    Alternatively, a hemacytometer (APPENDIX 3B) can be used to perform cell counts (also see http://www.animal.ufl.edu/hansen/protocols/hemacytometer.htm).

Analyze data

28. Determine cell counts:

    Cell count: Number from Coulter Counter (1:500 dilution)
    Cells $\times$ 10^6: Average cell count/5000.

29. Determine PFC/ml in original suspension:

    PFC/ml = plaques per 0.1 ml of cell suspension $\times$ 10 $\times$ dilution factor.

30. Determine PFC/spleen:

    PFC/spleen = (PFC/ml) $\times$ 3 ml.

31. Determine PFC/10^6 spleen cells:

    PFC/10^6 spleen cells = (PFC/ml)/(cells $\times$ 10^6/ml).

MISHELL-DUTTON ASSAY: IN VITRO PLAQUE FORMING CELL RESPONSE TO SHEEP RED BLOOD CELLS

ALTERNATE PROTOCOL 1

The Mishell-Dutton assay analyzes the humoral immune response described in the Basic Protocol utilizing an in vitro system (Mishell and Dutton, 1967). Exposure to both the putative immunotoxic chemicals and the sRBC occurs in vitro. Critical conditions are required for the in vitro development of humorally mediated hemolytic responses against red blood cells by splenocytes. These conditions include the addition of 10% fetal bovine serum (FBS) and 50 µM 2-mercaptoethanol (2-ME) to the culture medium and a gentle agitation of the culture at 37°C for 5 days in a pressurized (6-psi) mixed-gas atmosphere. All of these conditions have been shown to help enhance the in vitro IgM response to sRBC.

Additional Materials *(also see Basic Protocol)*

Complete RPMI medium (see recipe)
50% (v/v) suspension of sRBC (unprocessed, from original sheep donor) in Alsever's solution (see recipe for Alsever's solution)
Blood gas mixture: 7% CO_2/10% O_2/83% N_2
DEAE-dextran (powder)

48-well tissue culture plates, sterile
Mishell-Dutton pressure box (CBS Scientific)
24 $\times$ 40–mm coverslips (~450 mg; Fisher)
100-ml glass bottle
100-ml Erlenmeyer flask
45°C water bath

Additional reagents and equipment for assessing cell viability (*UNIT 18.8* or *APPENDIX 3B*)

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

Isolate and culture spleen cells

1. Sacrifice mice (without prior exposure to the chemical of interest or immunization with sRBC) and remove spleens according to the method described in the Basic Protocol, steps 6 and 7.

   Use extra caution with respect to sterility. Sterilize surgical instruments with ethanol and complete the dissection process as quickly as possible.

   Typically spleens from three mice can be pooled and used for this experiment.

2. Working in a tissue culture hood and using aseptic technique, prepare a single-cell suspension of spleen cells in HBSS/HEPES (Basic Protocol, steps 10 and 11).

3. Centrifuge splenocytes for 10 min at 300 × *g*, 4°C.

4. Remove the supernatant and resuspend the splenocyte pellet in 3 ml of complete RPMI medium. Mix the solution using a 5-ml pipet to aid in breaking up the pellet into single cells. Keep cells on ice between steps.

Prepare sRBC for use in immunizing the splenocytes

5. Place 0.5 ml of a 50% suspension of sRBC in Alsever's solution in a 15-ml conical tube. Add 5 ml HBSS, pH 7.0, then centrifuge at 5 min at 1050 × *g*, 4°C.

   The sRBC used for immunization in this protocol are not processed as in Support Protocol 1. The same blood stock should be used for immunization and plaquing, however.

6. Remove the Alsever's/HBSS supernatant and wash the sRBC twice more, each time by adding 5 ml HBSS, pH 7.0, centrifuging again as in step 5, and removing the supernatant. Resuspend the final sRBC pellet in 1 ml of HBSS, pH 7.0.

7. Transfer a 2-μl aliquot of sRBC into an Accuvette II vial containing 10 ml of Isoton. Mix, then transfer a 100-μl aliquot to a second Coulter vial, also containing 10 ml of Isoton, and mix.

8. Set the Coulter Counter to a diameter range between 3.3 and 6.647 μm and count cells in the second (1:500,000) dilution. Multiply the number displayed on the Coulter counter by 5×10^5 to obtain the sRBC concentration in cells/ml.

   Duplicate counts should be taken for both splenocytes and sRBC to ensure that accurate cell counts are obtained.

Prepare splenocyte/sRBC mixtures

9. Add 20 μl of the splenocyte suspension from step 4 to an Accuvette II vial containing 10 ml of Isoton. Add two drops of Zap-O-Globin to lyse endogenous red blood cells that may be present and count the cells in the Coulter Counter using the same settings as for the sRBCs. After the counts are taken, multiply the number displayed on the Coulter counter by 5×10^3 to obtain the final cell concentration (cells/ml).

10. Add an appropriate volume of complete RPMI medium to the splenocytes to yield a final splenocyte concentration of 1×10^7 cells/ml.

    The calculation is: [(x cells/ml)/(1×10^7 cells/ml)] × present volume in ml = final volume in ml needed to yield 1×10^7 splenocytes per ml.

11. Add an appropriate aliquot of sRBC to 1 ml of HBSS to yield a final sRBC concentration of 2.5×10^8 cells/ml.

    The calculation is: [(2.5×10^8 cells)/(ml/x cells/ml)] × 1000 μl = μl of sRBC to add to 1 ml of HBSS to yield 5×10^6 sRBC per 20 μl.

	1	2	3	4	5	6	7	8
A	X	X	X	X	X	X	X	X
B	X	conc 1	conc 1	background	background	positive control	positive control	X
C	X	conc 2	conc 2	vehicle	vehicle	conc 3	conc 3	X
D	X	conc 3	conc 3	X	conc 2	conc 2	X	X
E	X	positive control	positive control	conc 1	conc 1	vehicle	vehicle	X
F	X	X	X	X	X	X	X	X

Figure 18.11.3 Example of experimental plate setup for Mishell-Dutton assay. Wells marked with X receive HBSS medium only.

12. Add 500 μl of the 1×10^7 cell/ml splenocyte suspension per well of a 48-well tissue culture plate using a 1-ml automatic pipettor (total of 5×10^6 splenocytes/well). Leave the outermost wells empty. Using an automatic pipettor with a 50-μl tip, add 20 μl of the 2.5×10^8 cell/ml sRBC suspension per well (total of 5×10^6 sRBC, for a 1:1 ratio of splenocytes to sRBC). Omit the sRBC from 1 to 2 wells (leaving them to contain only the splenocytes), which will be used as no-blood (no sRBC) controls to test for background IgM activity.

Vary the distribution of the control and sample wells on the plate to avoid confounding results due to location of the samples within the plate.

Expose the cells to test chemical

13. Add the test chemicals to the appropriate wells in a volume of no more than 20 μl.

A good experimental design will consist of four or more replicates per group with several concentrations examined for the chemical of interest. Positive controls can include 0.1 μM ferrous sulfate (Ban et al., 1995).

A sample experimental plate is illustrated in Figure 18.11.3.

14. Pipet 500 μl of complete medium without cells into the outer of wells (see Fig. 18.11.3) to form a buffer so that dehydration of the samples does not occur.

15. Place 48-well plate in the upper tray of a Mishell-Dutton pressure box and place a small petri dish containing moist gauze in the lower tray. Seal the box and flush an atmosphere of 7% O_2/10% CO_2/83% N_2 through the chamber. Pressurize the chamber to 6 psi with the same gas mixture and close valves.

16. Incubate the culture in the Mishell-Dutton box at 37°C for 5 days.

Prepare dishes for plaque assay

17. Label 100-mm petri dishes with a marking pen to correspond to each well of the culture to be plaqued.

Four culture wells will be plaqued in each petri dish.

18. For each petri dish, place four 24 × 40–mm cover slips on a working surface.

Prepare agar

19. Add 0.1 g DEAE-dextran to 50 ml HBSS, pH 7.0 (for a final DEAE-dextran concentration of 0.2% w/v), in a 100-ml glass bottle that has been warmed to 45°C. Mix and allow the DEAE-dextran to dissolve completely in the HBSS.

20. Add 0.7 g Bacto Agar to 50 ml of HBSS, pH 7.0 (for a final agar concentration of 1.4% w/v), in a 100-ml Erlenmeyer flask.

21. Dissolve the agar by heating the HBSS-agar mixture in a microwave oven set on high for 3 min. Observe the bottle and stop the microwave if the mixture appears to be boiling over.

22. Mix the agar by gently swirling the bottle, then return the bottle to the microwave oven and continue heating until the agar is completely dissolved. Make sure the agar is completely dissolved before use.

    CAUTION: *Use a heavy glove or several folded paper towels to handle the bottle to avoid being burned.*

23. Pour the dissolved agar/HBSS mixture into the bottle containing the 45°C HBSS/DEAE-dextran from step 19. Swirl the liquid in the bottle to ensure that the two solutions are homogenously mixed. Keep in a 45°C water bath.

    The mixture should become cloudy.

24. Place one 12 × 75–mm test tube for each sample in a rack in a 45°C water bath. Add 400 µl of the HBSS/agar/DEAE-dextran solution to each tube. Wait at least 15 min before plaquing so that the temperature of the agar can equilibrate with that of the water bath.

Prepare sRBC

25. Prepare the sRBC for plaquing by adding 5 ml of a 50% suspension of sRBC (unprocessed; from original sheep donor) in Alsever's solution to a 15-ml conical tube containing 10 ml HBSS.

26. Centrifuge the cells 10 min at 1050 × *g*, 4°C. Remove the Alsever's/HBSS supernatant and add 10 ml HBSS, centrifuge again as before, and remove the supernatant.

27. Resuspend the final sRBC pellet in an equal volume of HBSS for a final concentration of ~1.2×10^{10} cells/ml.

28. Resuspend lyophilized GPC according to the manufacturers' instructions. Dilute 1 part resuspended GPC with 3 parts HBSS before plaquing.

    The complement is temperature sensitive and needs to be kept on ice at all times. 1.5 ml of complement should be enough to plaque 60 culture wells.

Perform plaque assay

29. Slowly depressurize the Mishell-Dutton box by opening a valve on the side of the box (rapid depressurization will kill the cells). Open the front plate on the box and remove the 48-well culture plate.

30. Place the culture plate on ice and mix the cells in each well by pipetting up and down with an automatic pipettor set for 100 µl, using a new pipet tip for each treatment group.

31. To determine the volume of sRBC necessary for plaquing, add 20 to 30 µl of the sRBC to one of the test tubes containing 400 µl of the agar mixture. Remove the test tube from the water bath, vortex briefly, then place a 200-µl aliquot in a 100 × 15–mm petri dish.

32. Breathe on a 24 × 40–mm cover slip (weighing ~450 mg) to moisten it slightly and drop on the sRBC-agar aliquot. Allow the agar to set for 5 min and then examine for color contrast.

In the PFC assay described in subsequent steps, plaques form due to lysis of the sRBC. This results in a clear spot contained within a background of agar (Fig. 18.11.2). A background color dark enough to allow plaques to be seen but not dark enough to obscure them is ideal. If the contrast is too dark, fewer sRBC should be added, and if it is too light, then additional sRBC should be added. The volume of sRBC determined in this way gives the best color contrast for plaquing the cultured splenocytes.

33. Prepare each sample by adding the following items to a 12 × 75–mm test tube kept in a 45°C water bath:

    400 µl HBSS/agar
    20 to 30 µl containing optimal number sRBC for plaquing (can be added to several tubes at once)
    100 µl cultured splenocytes (add to one tube at a time)
    25 µl GPC (from step 28; add after tube has been removed from water bath).

34. Place a 200-µl aliquot of the mixture from the test tube in a 100-mm petri plate. Before the agar has solidified, breathe on a 24 × 40–mm coverslip (weighing ~450 mg) to moisten it slightly and drop on the sRBC-agar aliquot.

35. When the agar has solidified, place plates in a 37°C incubator for 3 hr.

Count splenocytes

36. While the PFC plates are incubating, add 100 µl of the original splenocyte suspension from step 27 to 10 ml Isoton in an Accuvette II vial.

37. Add two drops of Zap-O-Globin II just prior to Coulter counting. Set the Coulter Counter to a diameter range between 3.3 and 6.647 µm and count cells. Record the cell number and use this to calculate PFC/10^6 splenocytes after plaques have been counted (see step 40).

38. Determine splenocyte viability by propidium iodide exclusion method (*UNIT 18.8*) or other methods such as trypan blue exclusion (*APPENDIX 3B*).

Count plaques and calculate PFC

39. Following the 3-hr incubation time (step 35), count the plaques using a plaque viewer or an inverted microscope

    Each clear circular spot in the agar (hemolysis of sRBC) represents one antibody- producing B cell and is counted as one plaque (Fig. 18.11.2).

40. Calculate the PFC per 10^6 cells and per 10^6 viable cells.

    Cell counts = number from Coulter Counter (1:100 dilution)
    Viability = number of stained cells/total number of cells
    Cells × 10^6 = average cell count/1000
    PFC/10^6 viable cells = (average PFC × 50)/(cells × 10^6) × (viability/100)
    PFC/well = average PFC × 5.

IN VIVO CHEMICAL EXPOSURE/IN VITRO PFC RESPONSE TO sRBC

ALTERNATE PROTOCOL 2

Certain chemicals may require metabolic activation before an immunotoxic effect can be identified. In this scenario, a metabolite and not the parent compound may be eliciting the effect. Analysis of these types of compounds cannot be achieved by the standard Mishell-Dutton culture system. An alternative approach is to expose animals to the test chemical in vivo and then immunize the splenocytes in vitro. This protocol combines methods of the first two protocols by exposing animals in vivo and then processing and analyzing the spleen in vitro. Follow methods from Alternate Protocol 1 (Mishell-Dutton assay) starting at step 1, except remove the spleen from an animal that has been previously exposed to the chemical of interest. The animal should not be immunized with sRBC in vivo and no chemicals or toxins should be added in vitro.

ALTERNATE PROTOCOL 3

IN VITRO PLAQUE-FORMING CELL RESPONSE TO sRBC WITH A METABOLIC ACTIVATION SYSTEM

A modification of the plaque assay can be used to examine the effect of a metabolized chemical on the PFC response to sRBC in an entirely in vitro system. When a metabolite and not the parent compound is required for the immunotoxic effect, it is necessary to add a metabolic activation system to the culture. The S-9 microsomal fraction prepared from mouse livers is commonly used as an in vitro metabolic simulator (Tucker et al., 1982; *UNIT 3.1*). S-9 will catalyze an enzymatic reaction that converts the parent compound into its metabolites in an in vitro system. It is important to note that this system may not work for all chemicals.

No particular class of immunosuppressive chemicals has been found to require metabolic activation, but examples of compounds that do require metabolic activation include cyclophosphamide, nicotine, casein, carbon tetrachloride, dimethylnitrosamine, cyclosporin, rapamycin, and aflatotoxin.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

20 mg/ml stock S-9 fraction of pooled female mouse livers (see *UNIT 3.1*)
12-well culture plate

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

1. Prepare the splenocytes and sRBCs as described in Alternate Protocol 1, steps 1 to 11.
2. Set up a culture of 1×10^7 splenocytes in 1 ml of complete medium per well of a 12-well culture plate.
3. Add chemical of interest and S-9 in a 0.1 ml volume.

   The optimal concentration of S-9 may range from 0.5 to 1.5 mg/ml and will need to be determined prior to the study.
4. Incubate for 1 hr at 37°C in Mishell-Dutton chamber under standard culture conditions (see Alternate Protocol 1, step 15) to allow for the metabolism of the chemical of interest.
5. After 1 hr, transfer the contents of each well to a sterile 12 × 75–mm snap-cap tube and centrifuge 10 min at 2000 × *g*, 4°C.
6. Decant the supernatant and resuspend the pellet in 1 ml complete medium. Transfer 500 µl of the suspension to appropriate wells of a 48-well culture plate and add sRBC as described in step 12 of Alternate Protocol 1.
7. Continue with steps 13 to 40 of Alternate Protocol 1.

ALTERNATE PROTOCOL 4

MEASURING PLAQUE-FORMING CELL RESPONSE TO T-INDEPENDENT ANTIGENS

Although not as commonly used, other antigens in addition to sRBC have been characterized for their stimulation of B cells. Lipopolysaccharide (LPS) and dinitrophenyl (DNP-Ficoll) stimulate B cells in the absence of T cells and are thus termed T-independent (TI) antigens. LPS and DNP are just two of the many identified TI antigens; it is these that will be discussed in this protocol. The mechanism for antibody production by LPS is related to its potent mitogenic activity and has been designated as a type 1 T cell–independent antigen, or polyclonal activator. DNP-Ficoll is an example of a type 2 T-independent antigen because its mechanism of B cell activation is not related to its mitogenic potency.

The DNP response requires the participation of B cells and macrophages (Mond, 1982), while the response elicited by LPS only requires the participation of B cells (Skidmore et al., 1975). The peak response of B cells stimulated by DNP occurs 4 days after addition of B cells in an in vitro system, while the peak response of LPS occurs at 2 to 3 days after addition to B cells in an in vitro system. These assays can provide information on the specific cell type affected by the chemical of interest. If one can identify suppression of IgM production in the T cell–dependent in vitro system, but not in either of the T cell–independent systems, it can be inferred that T cells are the cell type being affected. Analysis of the PFC response using multiple types of antigens can provide more insight about the specific mechanisms of regulation.

Enumeration of the PFC response to DNP-Ficoll and LPS using the Mishell-Dutton assay is performed as described in Alternate Protocol 1, with a few minor changes.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

10 mg/ml stock solution of LPS from *Escherichia coli* (strains 0128, 0111, and 055 are commonly used; Difco) *or* 5 μg/ml DNP-Ficoll (Biosearch Technologies; *http://www.biosearchtech.com/)*
50% (v/v) suspension of haptenated sRBC in HBSS, pH 7.0 (Support Protocol 2; Holsapple, 1995)

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

1. Omit steps for the preparation of sRBC for immunization (Alternate Protocol 1, steps 5 to 8 and 11). In step 12, add DNP-Ficoll at 0.05 μg/well, LPS at 50 μg/well, or other T-independent antigen(s) to the splenocyte culture instead of sRBC.
2. In the steps for preparing sRBC for plaquing (Alternate Protocol 1, steps 25 to 27 and 31), substitute haptenated sRBC. In step 33, add haptenated sRBC in quantity optimal for plaquing.
3. Measure DNP-Ficoll plaques on day 4 of the culture and LPS plaques on day 2 or 3 instead of day 5.

PREPARATION OF sRBC FOR IMMUNIZATION

SUPPORT PROTOCOL 1

Freshly collected sheep blood must be washed and diluted prior to use for immunization.

Materials

Sheep as source of blood
Alsever's solution (see recipe)
Hanks' balanced salt solution, pH 7.0 (see recipe)
Isoton (Beckman Coulter)
15- or 50-ml conical polypropylene centrifuge tubes
Tabletop centrifuge
Accuvette II vials (Beckman Coulter)
Coulter Counter (Beckman Coulter)

1. Collect 40 to 80 ml sheep blood and prepare a 50% (v/v) suspension in Alsever's solution ~1 week before its intended use. Store at 4°C.

   Blood should not be collected more than 3 weeks before use.

2. Using sterile technique, remove the amount of diluted blood necessary for the immunizations and transfer to a 15- or 50-ml conical centrifuge tube.

   A quantity of sRBC equivalent to 15 to 20 ml blood is needed to immunize 100 to 120 mice.

3. Wash blood by adding 2 vol of HBSS, pH 7.0, then centrifuging 10 min at 1050 × *g*, 4°C.
4. Using a Pasteur pipet, remove the Alsever's/HBSS supernatant along with the buffy coat (layer of white cells between the supernatant and pellet of red cells).
5. Wash twice more, each time by adding 2 vol of HBSS, pH 7.0, to the red cells, centrifuging again as in step 3, and removing the supernatant.
6. After last wash, resuspend the pellet in 10 ml HBSS.
7. To count sRBCs, dilute cells 1:500 in Isoton (20 µl cells in 10 ml Isoton), carefully wiping the outside of the pipet tip to remove excess cells before adding the blood to the Isoton, to ensure accurate sRBC counts. Mix well and dilute the previous dilution 1:100 in Isoton (100 µl in 10 ml Isoton) in an Accuvette II vial. Mix well.
8. Set the Coulter Counter to a diameter range between 3.3 and 6.647 µm and count 0.1 ml of the cell suspension.

   Dilution factor is 5×10^5: 1:500 × 1:100 × 10 (because 0.1 is sampled). If the cell count on the machine is 11.982, the cell number is $11{,}982 \times 5 \times 10^5 = 5.9910 \times 10^9$/ml.

   A hemacytometer (APPENDIX 3B) may be used for counting in lieu of a Coulter Counter.
9. Dilute cells to 3.75×10^8/ml in HBSS, pH 7.0, for immunizing B6C3F1 mice.

   Injecting 0.2 ml i.v. gives 7.5×10^7/mouse.

   Cell counts: 1:500 dilution
   Dilutions: cells/ml = average cell count × 500,000
   Stock sRBC needed for mice: 3.75×10^8 cells/ml × (final volume).

SUPPORT PROTOCOL 2

PREPARATION OF HAPTENATED sRBC

Haptenated sRBC are used for assessing the PFC response to T cell–independent antigens. This procedure is based on that of Trump (1972). The hapten that is transferred to the sRBC in this protocol is the trinitrophenyl (TNP) moiety. TNP serves as a "universal antigen" and can be recognized by multiple forms of antibody due to its simple structure and the possibility of cross-reactivity. For this reason, these haptenated sRBC can be used to detect the PFC response to either of the T-independent antigens used in Alternate Protocol 4—DNP or LPS.

Materials

Sheep as source of blood
Alsever's solution (see recipe)
Hank's balanced salt solution (HBSS), pH 7.0 (see recipe)
Cacodylate buffer: dissolve 44.8 g cacodylic acid in 1 liter distilled H_2O and adjust pH to 6.9 with HCl
0.015 M picryl sulfonic acid in cacodylate buffer
5.85 mM glycylglycine in cacodylate buffer
50-ml conical polypropylene centrifuge tubes
Refrigerated centrifuge
Parafilm
Platform rocker

1. Collect 40 to 80 ml sheep blood in Alsever's solution no more than 3 weeks before its intended use. Store at 4°C.
2. Place 5 ml of the sheep blood in Alsever's solution in a 50-ml conical tube. Add 20 ml HBSS, pH 7.0, and centrifuge 10 min at 300 × *g*, 4°C.

3. Remove the Alsever's/HBSS supernatant along with the buffy coat (layer of white cells between the supernatant and pellet of red cells). Add 20 ml HBSS, pH 7.0, to the sRBC pellet, centrifuge again as in step 2, and remove the supernatant.
4. Resuspend the sRBC pellet in 20 ml of cacodylate buffer. Centrifuge again as in step 2, then remove the supernatant and resuspend the pellet in 20 ml of 0.015 M picryl sulfonic acid.
5. Wrap the 50-ml tube in Parafilm, then secure it to the platform of a platform rocker and rock 10 min at 4 to 5 rpm at room temperature.
6. Centrifuge sRBC suspension as in step 2, and pour off the yellowish supernatant. Resuspend the pellet in 50 ml of 5.85 mM glycylglycine (dissolved in cacodylate buffer).
7. Centrifuge sRBC again as in step 2, remove supernatant, and resuspend pellet in 15 ml cacodylate buffer.
8. Centrifuge as in step 2 and remove supernatant. Wash sRBC three times, each time by adding 20 ml HBSS, pH 7.0, centrifuging again as before, and removing the supernatant.
9. Resuspend pellet at 50% (v/v) in HBSS, pH 7.0. Store the haptenated blood at 4°C and use up to 10 days after being prepared.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Alsever's solution

20.5 g dextrose (114 mM final)
7.9 g sodium citrate·2H_2O (27 mM final)
4.2 g NaCl (71 mM final)
H_2O to 1 liter
Adjust to pH 6.1 with 1 M citric acid and filter sterilize
Store indefinitely at 4°C

Hank's balanced salt solution (HBSS), pH 7.0

Add 10 ml of 10× HBSS (e.g., Cellgro) to a sterile 100-ml bottle in a tissue culture hood. Add 90 ml water to bring the solution to isotonicity. Add ~10 µl of 1 N NaOH to bring the solution to pH 7.0. Filter sterilize through a 0.22-µm filter. Store up to 1 month at 4°C.

pH should be adjusted before filtration to ensure sterility.

HBSS/HEPES

Add 50 ml of 10 × HBSS (e.g., Cellgro) to a sterile 500-ml bottle in a tissue culture hood. Add 7.5 ml of sterile 1 M HEPES (Invitrogen) to buffer the solution between pH 7.2 and 7.4. Add 445 ml of sterile water to bring the solution to isotonicity. Filter sterilize through a 0.22-µm filter. Store up to 1 month at 4°C.

2-Mercaptoethanol, 50 mM

Add 35 µl of concentrated 2-ME to 10 ml of RPMI-1640 medium in a 15-ml conical tube and vortex. Store up to 1 week at 4°C.

RPMI-1640 medium, complete

Add 90 ml of 1× RPMI medium (e.g., Invitrogen, Sigma) to a sterile 100-ml bottle in a tissue culture hood. Add 1.35 ml of 1 M HEPES buffer and 100 μl of 50 mg/ml gentamicin (both available from Invitrogen or Sigma). Thaw fetal bovine serum (FBS; *APPENDIX 3B*) and add to the medium to give 10% (v/v) final concentration (heat inactivation of FBS is not necessary). Finally, add 100 μl of 2-mercaptoethanol (see recipe) for a final concentration of 50 μM. Store up to 1 week at 4°C.

COMMENTARY

Background Information

Primary antibody response

Development of a primary antibody response requires the sequential orchestration of various cell populations. The characterization of the cellular interactions in this response was facilitated by the development of an in vitro immunization technique. Mishell and Dutton (1967) developed a method for evaluating the ability of dissociated spleen cells to produce IgM antibody–forming cells against the antigen sRBC following in vitro immunization. The in vitro induction of a primary IgM PFC response to a T-dependent antigen was demonstrated to require the interaction of three subpopulations in the spleen. The macrophage is the accessory cell responsible for initiating the response by processing the antigen and presenting it to the T cells. The T helper (T_H) lymphocytes regulate the B cell response by production of soluble proliferation and differentiation factors and cognate interactions. The B cell produces antibody with high binding specificity for the antigen. Generation of a primary antibody response requires two stages. The first involves initiation of the immune response by the antigen-presenting cells, and the second stage is the activation of antigen-specific B cells from their resting state via cytokines and cell-cell contact to proliferate and differentiate to a plasma cell, which secretes antigen-specific antibodies.

The first antibodies to be produced in a humoral immune response, in either an in vivo or in vitro system, are always IgM. These early IgM antibodies are produced before B cells have undergone somatic hypermutation, and therefore they tend to be of low affinity. Upon stimulation by antigen, the B cell will differentiate into a plasma cell producing large amounts of secreted IgM. Some cells will undergo a class switch during which a rearrangement of the DNA will occur, ultimately producing additional antibodies (IgG, IgE, or IgA). Upon secondary induction (i.e., the secondary response), these B cells will differentiate into plasma cells expressing the new isotype. Most commonly, this results in a switch from an IgM response (primary/direct response) to an IgG response (secondary/indirect response; Abbas, 1997).

Although not described in this unit, secondary immune responses (IgG) can also be evaluated using the PFC assay (North and Maizels, 1977). The IgG response will peak ~10 days after a secondary immunization. Keyhole limpet hemocyanin (KLH) is a T-dependent antigen that elicits a strong IgG response and it can also be useful in the evaluation of the secondary response (Herscowitz et al., 1975).

Alternative methods

The enzyme-linked immunosorbent assay (ELISA; *UNIT 18.7*) is another method that has been used to measure IgM and IgG antibody production in mice. The basic design depends on immobilization of the specific antigen (sRBC) on a modified plastic surface. Serum from the immunized animal is added to the plate. sRBC-specific antibodies bind to the antigen coated on the plate surface and unbound antibodies are washed away. A secondary anti-mouse IgM or IgG antibody that is covalently bonded to an enzyme is added, and a color change occurs upon addition of a substrate. The colored product provides a quantifiable measure of the antibody response. The historical background for use of the PFC assay in the identification of immunotoxic agents is considerably greater than for the ELISA. The comparability of the ELISA and PFC assays with respect to sensitivity in the detection of immunotoxicants has been demonstrated for numerous xenobiotics, such as the well known immunotoxicants benzo[a]pyrene and cyclophosphamide. The major difference between the two assays is that the PFC assay is organ specific, while the ELISA uses total serum IgM or IgG. The ELISA is also capable of recognizing isotypes of certain antibodies while the PFC assay cannot. Although not very common, it is possible for a chemical to induce a response in one assay but not the other (Wilson et al., 1999).

Cell separation/reconstitution

Given that the PFC response to sRBC is critically dependent on the cellular cooperativity of multiple cell types, deleting or impairing any of the necessary cells will eliminate or decrease the PFC response to sRBC. Using the Mishell-Dutton assay, cell separation/reconstitution studies can be used to identify target cell toxicity. This type of assay requires two phases. In the first phase, it is determined if a xenobiotic primarily targets adherent (macrophage) or nonadherent cells (B and T cells). If the target cell can be identified, in the second phase, there is a reconstitution of this cell type (isolated from a naive or control mouse) back into the culture. The PFC response will be reanalyzed to see if the immunosuppressive response is eliminated. Macrophages can be removed from a spleen cell suspension by capitalizing on their ability to adhere to plastic. This can be achieved by preincubation steps in plastic petri dishes or the passage of the spleen cell suspension through a Sephadex column. Both T and B cells can be removed from a cell suspension by negative selection using antibodies against their respective surface markers. Following incubation with the antibody attached to magnetic beads, the suspension is passed though a magnetic column where the specific cell type will adhere. These cell separation methods can be used in conjunction with analysis of the PFC response using T-independent or polyclonal antigens to help characterize the specific cellular participants involved in the immune modulation as a result of exposure to a particular xenobiotic.

Critical Parameters and Troubleshooting

All of the experiments described in this unit are extremely sensitive to experimental conditions and there are many factors that can affect the outcome. Because IgM needs complement for proper lysis of the sRBC, all experiments rely on this component (Nielsen et al., 2000). Complement is temperature sensitive and needs to be kept on ice at all times. The temperature of the medium and water bath are also critical. If cells are added to the agar before it has equilibrated to the temperature of the water bath, they could be heat shocked and die. It is therefore extremely important to monitor the temperature of the water bath to maintain the designated temperature.

It is also very important to process the spleens as quickly as possible. This is especially critical for the in vitro assays. The intact spleens or cell suspensions used for the in vivo assay can be kept at 4°C for 24 hr and the PFC response will still be observed upon plaquing, although the response may be reduced. The age of the sheep blood is also critical for both in vivo and in vitro assays, but the in vivo assay is more sensitive. The blood cells need to be intact for optimal IgM response in vivo but not in vitro. Blood that has aged for too long will not work for the in vivo assay. Use blood that is no older than 3 weeks for the immunizations. The age of the blood is equal to the time since it was collected from the sheep. The age of the blood used to prepare the sRBC for plaquing is not as critical, and blood that has aged over a month has been shown to elicit a PFC response. It is also important to wash the blood that will be used for immunizations with HBSS that has been brought to a pH of 7.0 with NaOH and which is not buffered with HEPES. HEPES will interfere with normal antigen processing of the sRBC. The age of the mouse can also influence the results of these experiments. Mice between 8 and 11 weeks old are acceptable for use with this immunotoxicological assay. Although this unit focuses only on studies done on mice, rats can also be used. Minor modifications of the protocols are required for studies done on rats (Smialowicz et al., 1994).

The in vitro assay requires additional components because the splenocytes are cultured for 5 days. Fetal bovine serum (FBS) is a very important component because it helps to enhance the in vitro PFC response to sRBC by providing a variety of necessary components. Cells will not survive without it because it is a source of growth factors. It is important to screen several batches of FBS and identify one that yields the greatest response because not all batches will work the same. 2-ME is also critical in the in vitro assay to enhance the PFC response to sRBC because it aids in the proliferation of the B cells. DEAE-dextran is critical in both the in vivo and in vitro assays because it inactivates endogenous inhibitors of complement that may be present in the Bacto Agar.

The housing and care of the mice has also been shown to greatly influence the outcomes of these experiments. The mice need to be housed and maintained in a pathogen-free environment with exposure to minimal amounts of stress. Stress has been shown to reduce the PFC response to sRBC. If male mice are used they should be housed individually to prevent fighting.

Anticipated Results

Based on data pooled from several different laboratories, the in vivo PFC/spleen ($\times 10^3$) response has been shown to range anywhere from about 200 to 500, while the PFC response/10^6 cell ranges from 800 to 3500 (Zimecki and Wieczorek, 2001; Bin-Hafeez et al., 2003; Keil et al., 2004). The results are typically presented both ways to determine the relative contribution to the effects of a chemical on the cellular components of the spleen. In most cases, if a chemical elicits an immunotoxic effect, both parameters will be decreased, but it is possible for a chemical to produce a significant reduction in the PFC/spleen but not in the PFC/10^6 cell. In this case, the splenocyte number is probably reduced, but the remaining cells are still immune competent.

Expected responses for the in vitro assay can vary depending on the type of antigen used. T-dependent antigens typically give a response of 300 to 500 PFC/10^6 cells, T-independent antigens give a response between 200 to 600 PFC/10^6 cells, and polyclonal antigens give a response between 300 to 800 PFC/10^6 cells.

Time Considerations

In vivo PFC response

The time requirements for this experiment will vary depending on how many animals are used for a study, what endpoints are being analyzed, and the specific chemical being investigated. Length of study can vary depending on how many doses of the chemical of interest the mice will receive and how much time will elapse between doses. Immunization with sRBC usually requires ~1 hr for 25 to 30 mice. Once the spleens are removed from the mice, they must be made into single-cell suspensions. This step will require about 30 to 45 min for an average 25-mouse study. Preparation of the sRBC and agar can be done before the spleens are removed. This step can take 45 to 60 min. The time requirements for plaquing the assay will depend on the number of animals used for the study. It can be estimated that it will take 1 hr to plaque the splenocytes from 25 to 30 animals. Plan on devoting an entire day to running this assay from start to finish.

Alternate Protocols 1 and 3

Time considerations are reduced for these protocols because the number of animals used is reduced, and the dosing of the animals is eliminated. A time requirement of 1 to 2 hr can be expected for setting up the culture. After the 5-day incubation, it will take ~1 hr to prepare for the plaquing and 1 to 2 hr to plaque the assay. Plan on devoting an entire day to running this assay from start to finish. When a metabolic activating system (MAS) is used, several additional hours will be added to the total time it takes to complete the experiment. One must take into consideration the preincubation time, the additional time needed for centrifugations, and time needed for addition of the MAS.

Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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