Adriamycin Nephropathy in BALB/c Mice

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Chronic proteinuric renal injury is a major cause of end stage renal disease. Adriamycin nephropathy (AN) is a murine model of chronic proteinuric renal disease whereby chemical injury is followed by immune and structural changes that mimic human disease. This unit describes the method of AN induced by a single injection of adriamycin (ADR) in BALB/c mice. After the initial toxic injury, an immune-mediated chronic proteinuric renal disease that resembles human focal segmental glomerulosclerosis develops. The clinic pathological features of AN are nephrotic syndrome, focal glomerulosclerosis, tubular injury, and interstitial compartment expansion with mononuclear cell infiltrates that are composed largely of macrophages and T cells. © 2015 by John Wiley & Sons, Inc.

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BASIC PROTOCOL

Adriamycin (ADR) is a commonly used chemotherapeutic agent that produces significant tissue damage. Adriamycin nephropathy (AN) induced by ADR is an experimental rodent model of kidney disease that has been studied extensively and is a well-established model in research laboratories in both mice and rats (Wang et al., 2006; Zheng et al., 2006; Polhill et al., 2012). This model is characterized by severe proteinuria and the development of chronic kidney disease. It is associated with macrophage and lymphocyte infiltration reflecting an immune inflammatory response to initial injury. Recent studies further demonstrated that endothelial dysfunction and damage precede podocyte injury in ADR-induced nephropathy (Sun et al., 2013).

AN only occurs amongst specific inbred mouse strains, such as BALB/c and 129S1/SvImJ, whilst other strains (such as C57BL/6J) are relatively resistant. Studies have suggested that this selective susceptibility to kidney injury is caused by certain gene mutations (Papeta et al., 2010). AN has several strengths as an experimental model of kidney disease. It is a highly reproducible model of renal injury and has acceptable mortality and morbidity (Lee and Harris, 2011). Different therapeutic strategies can be used to treat the animals before or after ADR injection.

Here we describe the method of generating AN in BALB/c mice in our laboratory (Basic Protocol). After AN induction, development of clinical disease is monitored by an increase in proteinuria, a rise in serum creatinine, and a loss of body weight. Serum and organs are harvested at 4 to 5 weeks after ADR administration, in order to assess renal function, structural injury, and degree of infiltrating immune cells within the kidney.
An is induced in BALB/c mice by injection of a single dose of ADR via the tail vein. The animals are usually able to tolerate this injection without significant immediate morbidity/mortality but ultimately develop renal failure. Four to five weeks after ADR administration, blood, urine, and kidney samples are harvested for serum creatinine, urine protein, and histological assessment. RNA is isolated from mouse splenocytes for RT-PCR to assess the mRNA expression of cytokines. Flow cytometric analysis, histology, and morphometric evaluation and immunohistochemical staining are performed at the same time. Here we describe the method of AN induction commonly used in our laboratory.

**NOTE:** All protocols using live animals must first be reviewed and approved by an institutional Animal Ethics Committee and must follow officially approved procedures for the care and use of laboratory animals.

**Materials**

- Male BALB/c mice, 8 weeks old (20 g)
- Adriamycin (ADR; doxorubicin hydrochloride; 10 mg/5 ml per vial as a stock solution, Pfizer Australia)
- 10% neutral-buffered formalin (Sigma-Aldrich)
- Colorimetric assay kit (Bio-Rad, Hercules)
- Paraffin
- Periodic acid-Schiff (PAS) kit (Sigma-Aldrich)
- Optimal cutting temperature (OCT) compound (Sakura Fintek)
- Karnovsky’s fixative (see recipe)
- Nunc C96 Maxisorp MicroWell plates (Thermo Fisher Scientific)
- Multiskan EX Microplate photometer (Thermo Fisher Scientific)
- VITROS automated chemistry analyzer (Ortho Clinical Diagnostics)
- Scan Scope digital slide scanner (Aperio Technologies)
- Image J software (National Institutes of Health, Bethesda, Maryland)
- DeltaVision Core microscope (Applied Precision)
- 1-ml syringe
- 27-G needle
- Metabolic cages
- Water bottles
- 5-ml urine collection tubes

Additional reagents and equipment for euthanasia (UNIT 1.8; Donovan and Brown, 2006a), parenteral injections (UNIT 1.6; Donovan and Brown, 2006b), blood collection (UNIT 1.7; Donovan and Brown, 2006c), histology (UNIT 21.8; Hofman and Taylor, 2013), and flow cytometric analysis (Coligan et al., 2014).

**Induction of adriamycin nephropathy (AN)**

1. Maintain male BALB/c mice free of pathogens in a dedicated animal research facility.

   *Experiments are carried out in accordance with protocols approved by the institutional Animal Ethics Committee.*

2. Inject ADR via tail vein of each non-anesthetized mouse (9.6-9.8 mg/kg; UNIT 1.6; Donovan and Brown, 2006b). Inject saline for control group.

   *Mice are warmed up under a warming lamp for about 10 min or their tails are soaked in warm water for a couple of minutes. This is a useful method for tail-vein dilatation before injection. Avoid using alcohol, since it makes the tail vein vasoconstrict. Total volume of ADR mixture is 200 to 300 µl. A 1-ml syringe and a 27-G needle are used for this injection.*
As an experimental design example, if 8 male BALB/c mice are required to have AN (single dose: 9.8 mg/kg), then:

Add 1020 \( \mu l \) \((102 \times 10)\) of \( H_2O \) to 980 \( \mu l \) \((98 \times 10)\) of ADR stock solution (purchased from Pfizer Australia as a 10 mg/5 ml per vial stock solution; 2 mg/ml) and mix. Total volume of ADR solution: 2 ml.

Take 200 \( \mu l \) ADR solution and inject for one \( \times \) 20 g mouse via tail vein.

Take 220 \( \mu l \) ADR solution and inject for one \( \times \) 22 g mouse via tail vein.

The dose of ADR is critical for this model and dose testing is required when used for the first time in new strains of animal, animals of different ages, and new batches of ADR. A usual dose for 8-week old male BALB/c mice with body weight of 20 to 22 g is 9.6-9.8 mg/kg. Mice <8 weeks old with body weight <18 g are usually more sensitive to ADR. Different doses of ADR usage are required in different strains of animal to induce AN. For immune-deficient SCID mice, a dose of 4.8 mg/kg is required. For male Wistar rats, 5 mg/kg is used for induction of rat AN.

Be careful to avoid extravasation during the injection procedure. ADR is a toxic agent that produces significant tissue damage if injected outside of the vein. Special attention to any signs of extravasation should be made during injection. In the event of extravasation of ADR, failure to induce AN, mouse tail injury, and even tail tissue necrosis could result. Injection of ADR is performed with slow speed after the needle is confirmed to be inside the blood vessel. This is confirmed by observing if the blood comes back through the tip of needle.

3. Measure body weights daily until day 28 to day 35 after ADR administration. Harvest blood, spleen and kidney samples from each group of mice at day 28 to day 35 after ADR injection.

Assess renal function

4. Assess renal function by measuring urine protein and serum creatinine. Collect urine from each mouse for 16 hr prior to sacrifice. Measure urine volume, protein, and creatinine. Collect blood samples for serum albumin and creatinine by cardiac puncture or vena cava puncture (0.5 to 0.8 ml total blood by puncture; see UNIT 1.7; Donovan and Brown, 2006c) at day 28 to day 35.

5. Collect urine samples: set up a metabolic cage including sufficient water in a water bottle and a 5-ml urine collection tube for each mouse. Label tubes appropriately and place mice into individual metabolic cages for 16 hr. Withhold food and provide access to plain water.

6. Return mice to housing cages after each urine collection to access food and drinking water.

Accurate urine collection without contamination and animal injury is always a big challenge for researchers. For AN, mice are fasted and water deprived while in the metabolic cages for 16 hr to collect urine at baseline and week 4 to 5. Commercial metabolic cages are useful and convenient to use. Specific attention must be paid to their limitations, for example, water leakage and evaporation of urine during overnight collection.

Perform histology and morphometric evaluation

7. Remove kidneys rapidly to avoid tissue degeneration.

Reliable renal injury is observed in experiments where the kidney harvest is carried out on day 28 to 35 after ADR injection.
8. Fix sagittal slices of renal tissue in neutral-buffered formalin at room temperature for 24 hr and embed in paraffin for evaluation of pathology. Stain 5 µm slices with periodic acid-Schiff (PAS) kit and assess by light microscopy.

The remaining cortex of the same kidney is snap frozen in liquid nitrogen and used for RNA and immunohistochemistry.

9. Store kidney sections in specific fixatives for electron microscopy such as Karnovsky’s fixative.

10. In each section, assess and score a minimum of 10 consecutive fields at a magnification of X400. Estimate the degree of renal injury by evaluating the percentage of renal injury per field and grade on a scale of 0 to 4 (Mizuno et al., 1998).

In each biopsy, semi-quantitative scores from two blinded-trained researchers or doctors are used to evaluate the degree of renal injury.

Flow cytometric analysis
Mouse splenocytes were analyzed by flow cytometry. Antibodies used for flow cytometry included fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD25 and APC-conjugated anti-mouse-CD4 (BD Bioscience). Foxp3 intracellular staining was performed by using Anti-Mouse/Rat Foxp3 Staining Set PE (eBioscience, clone: FJK-16s) following manufacturer’s instructions. All samples were analyzed on a FACScan analyzer (Becton Dickinson, Mountain View, California). BD FACSDiva software was used for acquisition and analysis (Becton Dickinson, Australia).

REAGENTS AND SOLUTIONS
Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see APPENDIX 5.

Karnovsky’s fixative

- 10 ml 10% (w/v) paraformaldehyde (1% final)
- 35 ml 0.2 M sodium cacodylate (0.07 M final)
- 12 ml 25% (w/v) glutaraldehyde (3% final)
- 43 ml deionized H₂O

Adjust to pH 7.4 with 0.1 M hydrochloric acid. Store up to 1 week at 4°C.

COMMENTARY

Background Information
AN is induced in either mice or rats by a single intravenous injection of Adriamycin. After the initial toxic injury, chronic proteinuric renal disease develops that resembles human focal segmental glomerulosclerosis (Rangan et al., 1999). Researchers have utilized Adriamycin to induce nephropathy in mice or rats as a model of chronic proteinuric renal disease (Wang et al., 2000, 2001). The pathologic features of AN are severe nephrotic syndrome, focal glomerular sclerosis, and tubular injury with significant mononuclear cell infiltrates composed largely of macrophages and T cells (Rangan et al., 2001).

Critical Parameters and Troubleshooting
The strain of inbred animal is important for the induction of AN. AN occurs only in certain strains such as male BALB/c mice, male Wistar rats and 129S1/SvImJ mice, whilst other strains (such as C57BL/6J) are relatively resistant. Studies have suggested that this selective susceptibility to kidney injury is caused by certain gene mutations.

The dose of ADR is a critical parameter for this model. Therefore dose testing is required when used for the first time in new strains of animal, animals of different ages and new batches of ADR. Overdose will result in higher animal mortality and morbidity.
In contrast, lower doses will fail to induce disease in this model. A usual dose for 8-week old male BALB/c mice with body weights of 20 to 22 g is 9.6-9.8 mg/kg. Mice less than 8 weeks old with body weights less than 18 g are usually more sensitive to ADR. Different doses of ADR are required in different strains of animal to induce AN. For immune-deficient SCID mice, a dose of 4.8 mg/kg is required. For male Wistar rats, 5 mg/kg is used for induction of rat AN.

The technique of tail-vein injection is another important parameter for this model. Researchers with good animal handling experience perform this injection under a magnifying glass to find the best tail vein. Mice are warmed up under a warming lamp for about 10 min or their tails are soaked in warm water for a couple of minutes. This is a useful method for tail-vein dilatation before injection. Avoid using alcohol, since it makes the tail vein vasoconstrict; total volume of ADR mixture is 200 to 300 µl.

**Anticipated Results**

Four to five weeks after ADR administration, AN is induced in BALB/c mice by injection of a standard single dose of ADR. The animals are usually able to tolerate this injection without significant immediate
morbidity/mortality but ultimately develop renal failure. Electron and light microscopy demonstrate the glomerular damage, tubular injury, and a moderate interstitial infiltrate of inflammatory cells 5 weeks after ADR injection as shown in Figure 15.28.1. Blood, urine, and kidney samples are harvested for serum creatinine, urine protein, and histological assessment. RNA is isolated from mouse splenocytes for RT-PCR to assess the mRNA expression of cytokines. Flow cytometric analysis, histology, and morphometric evaluation and immunohistochemical staining are performed at the same time.

**Time Considerations**

Approximately 4 to 6 weeks are required to induce this model.

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**Literature Cited**


