STANDARD OPERATING PROCEDURES

DIVISION OF COMPARATIVE MEDICINE
UNIVERSITY OF SOUTH FLORIDA

TITLE: **Production of Polyclonal Antibodies In Rabbits**
SCOPE: All Animal Program Personnel
RESPONSIBILITY: Facility Manager, Animal Care Staff, All Animal Program Personnel
PURPOSE: To Outline the Proper Procedures for the Production of Polyclonal Antibodies in Rabbits

I. PURPOSE

1. To outline the proper procedures for the preparation of antigen emulsion, primary and booster immunizations, blood collection, and serum preparation for the production of antibodies in rabbits.

II. RESPONSIBILITY

1. The Veterinarians oversee all aspects of animal health.

2. The Facility Manager is responsible for scheduling, coordinating, and performing antibody production in rabbits.

III. INTRODUCTION

1. Polyclonal antibodies (PAbs) are important reagents utilized in a variety of experimental techniques (i.e., vaccines, immunoblotting, immunoprecipatation, and diagnostic tests) in many fields of biomedical research. Antigens (substances that stimulate antibody production by the body) are rarely sufficiently antigenic to directly induce a satisfactory immune response. They are usually administered in conjunction with adjuvants (substances that stimulate the immune response) to enhance the body's response to the antigen. Rabbits are commonly used for PAb production based on their size, ease of use, and strong immune response.

2. Historically, the single most commonly used adjuvant in the research setting has been Freund's adjuvant. Freund's adjuvants are available in two forms: Freund's Complete Adjuvant (FCA) which contains killed *Mycobacterium tuberculosis,* or Freund's Incomplete Adjuvant (FIA) without the additional bacterial component. The mycobacterial cell wall is a potent immune enhancer. FCA is an extremely potent inflammatory agent and may **only** be used for the primary immunization. **Only FIA should be used for booster immunizations after initial use of FCA**. Anaphylactic reactions can occur when FCA is used more than once and these reactions may be fatal. The preparation of the antigen emulsion is the same for both. FCA is known to commonly produce undesirable side effects. Granuloma formation, tissue necrosis and sloughing, abscessation and fever are routinely seen.

3. FCA is considered a human biohazard since accidental self-inoculation or splashing in the eye have been shown to cause painful inflammatory lesions and abscesses, hypersensitivity reactions, as well as sensitization to tuberculin which negates future skin testing. **Always** use gloves, gowns, and protective eyewear when handling FCA. **Always** use Luer-lock syringes when working with FCA. **Avoid** needle sticks: **Do Not** Re-Cap Needles!

4. Alternative adjuvants are now available that produce high antibody titers, in some cases exceeding those obtained with FCA, and with less severe inflammatory or toxic side effects. Many of these new adjuvants are suitable and highly effective for research use. A few examples include Hunter's TiterMax®, the RIBI adjuvant system® (RAS®), Monophosphoryl Lipid A®, and Adjuvax®. Hunter's TiterMax® and RIBI adjuvant system® are two adjuvants in common use. The Ribi adjuvant system® consists of monophosphoryl lipid A, synthetic trehalose dicorynomycolate, and cell wall skeleton, and recommendations of the manufacturer are followed (Ribi Immunochem Research, Inc., 406-363-6214). The Titermax® adjuvant system consists of a block copolymer CRL-8941, and recommendations of the manufacturer are followed (Hunter's Titermax 800-345-2987). Although Titermax® and RAS® are less toxic than FCA, similar precautions should be used when working with these two adjuvants.

IV. PRIMARY IMMUNIZATION PROCEDURES

1. The PI supplies the antigen. The animal care staff mixes the antigen and selected adjuvant aseptically according to the adjuvant manufacturer's instructions (usually in a 1:1 ratio). Maintain the syringes containing the antigen/adjuvant emulsion at temperatures appropriate for the adjuvant until used (4° C [40° F] for Freund's, room temperature for TiterMax®).

2. At least 2-4 female New Zealand White rabbits (2-4kg) should be used for each antigen. Female rabbits are generally preferred because they are more docile and are reported to mount a more vigorous immune response than males.
    a. Primary Immunizations should not be done in the animal housing room.
    b. Rabbits may be sedated to facilitate handling. To sedate the rabbit administer 10-25 mg/kg Ketamine + 1-3 mg/kg acepromazine IM in the caudal (quadriceps) muscles of the hind leg. Give the acepromazine first. The rabbit should be sedated, but not anesthetized. Allow approximately 15 minutes for the drugs to take effect.
    c. If phlebotomy and injections are performed with the rabbit awake, the rabbit should be securely restrained in a commercial restrainer or by an additional staff member.
    d. Always collect a pre-immunization blood sample (approximately 5-10ml) in a red top or serum separator vacutainer tube from the right or left ear veins or arteries before the primary immunization.

3. Once the rabbit is sedated or restrained, shave the dorsal thorax and lumbar area. The shaved area should be at least 4-5 square centimeters (2 square inches) to allow clear visualization of the injection sites and a clean work field. The dorsal neck region should be avoided since the skin in this area is commonly used for restraint.
    a. Aseptically prep the area by scrubbing with chlorhexidine scrub and rinsing with alcohol.

b. The antigen-adjuvant emulsion may be injected subcutaneously (≤0.2 ml per site at <12 sites per animal using a 23-25 gauge needle) or intradermally (≤0.1 ml per site at 10-12 sites per animal using a 25 gauge needle). Intraperitoneal, intravenous, or footpad injections are not acceptable routes of administration. FCA causes ulcerations when injected intradermally and should be given by the subcutaneous route of administration only.
c. The injection sites should be evenly separated over the dorsal thorax and lumbar area with at least 2 cm between sites and at least 3-4 cm from the spinal column to avoid accidental injection into the paraspinal muscles. This distributes the antigen over a larger surface area allowing for greater exposure to the immune system resulting in higher titers while reducing the incidence of severe local inflammatory response and abscessation.
d. Use a sharpie marker to draw circles about the size of a quarter around each injection site
e. Return the rabbit to its cage and monitor its recovery from sedation.
f. Make appropriate entries in the rabbit's medical record and on the ***Antibody Production Schedule*** (CMDC #011).
g. Monitor the injection sites daily for any type of reaction. Observation should include an assessment of the injection sites for tissue necrosis and abscess formation, as well as an evaluation of the animal's activity, food/water consumption and body condition. The attending veterinary staff should be notified immediately of any health concerns.
h. Care should be taken to avoid injection sites when handling the animal.

V. BOOSTER IMMUNIZATION PROCEDURES

1. The PI supplies the antigen. The animal facility staff mixes the antigen and selected adjuvant aseptically according to the adjuvant manufacturer's instructions (usually in a 1:1 ratio). Maintain the syringes containing the antigen/adjuvant emulsion at temperatures appropriate for the adjuvant until used (4° C [40° F] for Freund's, room temperature for TiterMax®). **Only** FIA may be used for booster immunizations after initial use of FCA.

2. Booster immunizations will be given according to the ***Antibody Production Schedule*** (CMDC #011).
    a. Booster immunizations may be done in the animal housing room.
    b. The rabbit does not need to be sedated for booster immunizations. The rabbit should be shaved for booster immunizations.

3. All booster immunizations are given IM in the caudal (quadriceps) muscles of the hind legs (≤0.5 ml per site at <3 sites per animal using a 23-25 gauge needle). Booster immunizations using TiterMax® may be administered SQ.
    a. The injection site should be swabbed with alcohol to remove debris and contaminants prior to injecting the antigen/adjuvant emulsion.
    b. When injecting into the quadriceps muscle care must be taken to avoid adjacent nerves and blood vessels as well as fascial planes of the muscle bundle.
    c. Return the rabbit to its cage.
    d. Make appropriate entries in the rabbit's medical record and on the ***Antibody Production Schedule*** (CMDC #011).
    e. Monitor the injection sites daily for any type of reaction. Observation should include an assessment of the injection sites for tissue necrosis and abscess formation or

self mutilation lesions of the rear legs and feet as well as an evaluation of the animal's activity, food/water consumption and body condition. The attending veterinary staff should be notified immediately of any health concerns.

VI. BLOOD COLLECTION PROCEDURES

1. Always collect a pre-immunization blood sample (approximately 5-10 ml) in a red top or serum separator vacutainer tube before the primary immunization to establish a serum baseline.

2. Additional blood samples will be collected according to the ***Antibody Production Schedule*** (CMDC #011).

3. Blood collection should not be done in the animal housing room.

4. Blood collection.
    a. Always use the marginal ear vein or central auricular artery for blood collection.
    b. The rabbit may be sedated to facilitate handling. Sedate the rabbit with 1-3 mg/kg acepromazine IM in the caudal (quadriceps) muscles of the hind leg. The rabbit should be sedated, but not anesthetized. Allow approximately 15 minutes for the drug to take effect. If phlebotomy and injections are performed with the rabbit awake, the rabbit should be securely restrained in a commercial restrainer or by an additional staff member.
    c. Swab the right or left ear with warm water and chlorhexidine solution to remove debris and contaminants.
    d. Apply tension to the ear and insert a 24-20g angiocatheter bevel-up into the blood vessel directed towards the base of the ear and attach the appropriate sized syringe (e.g.: 5-10cc).
    e. When appropriate volume has been drawn, withdraw the catheter, and apply direct pressure to the venipuncture site until the bleeding stops.
    f. Return the rabbit to its cage and monitor its recovery from sedation. Check for bleeding from the venipuncture site.
    g. The attending veterinary staff should be notified immediately of any health concerns.
    h. Make appropriate entries in the rabbit's medical record and on the ***Antibody Production Schedule*** (CMDC #011).

5. Label each tube of blood collected with the date, animal ID number, and PI.
    a. Allow the tubes to sit until the blood has clotted.
    b. After a clot has formed centrifuge the tubes for ten minutes at 2700 RPM.
    c. Put the tubes of blood in the refrigerator in Room 1301.
    d. Notify the PI that the samples are ready to pick-up.

6. When an adequate titer is detected, approximately 5.5 ml/kg (2.5 ml/lb) of blood can be collected every 14 days.

VII. EXSANGUINATION/EUTHANASIA

1. To anesthetize the rabbit administer 40-45 mg/kg Ketamine + 5-8 mg/kg Xylazine IM in the caudal (quadriceps) muscles of the hind leg. Allow approximately 15 minutes for the drugs to take effect. Alternatively, isoflurane (1-5%) may be administered by via an induction chamber and anesthesia is maintained with a mask/nose cone throughout the blood collection process.

2. Place the rabbit in dorsal recumbency.
    a. Attach a vacutainer adaptor needle to a vacutainer tube holder. Attach an 18 gauge X 2 inch needle to the vacutainer adaptor needle. Insert a red top or serum separator vacutainer tube into the holder and onto to the adaptor needle.
    b. Insert the needle directly into the heart (IC) after verifying the position of the heart by palpation and stethoscope.
    c. Replace and fill as many tubes as necessary to collect the blood.

3. When blood ceases to flow the rabbit is euthanized by injecting 1 ml/4.5 kg (1 ml/10 lbs) of Euthasol directly into the heart (IC) through the 18-20 gauge 1 ½ inch long needle using a 3 cc syringe, or alternatively by isoflurane overdose followed by bilateral thoracotomy.

4. Verify that the heart and respirations have stopped with a stethoscope.
    a. Make appropriate entries in the rabbit's medical record and on the ***Antibody Production Schedule*** (CMDC #011).
    b. Place the body in a red biohazard bag and store carcass in the freezer.

5. Label each tube of blood collected with the date, animal ID number, and PI.
    a. Allow the tubes to sit until the blood has clotted.
    b. After a clot has formed centrifuge the tubes for ten minutes at 2700 RPM.
    c. Put the tubes of blood in the refrigerator in Room 1301.
    d. Notify the PI that the samples are ready to pick-up.

VIII. REFERENCES:

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2. Hanly, W.C., J. E. Artwohl, and B.T. Bennett. "Review of Polyclonal Antibody Production Procedures in Mammals and Poultry", ILAR Journal, 37(3), pages 93-124, 1995.

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4. Leenaars, M., Hendriksen, C.F.M. "Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations", ILAR Journal, 46(3), pages 269-279, 2005.

Approved: **Date:**