A Protocol for Adjuvant-Induced Arthritis (AIA) in Rats

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BACKGROUND

In 1956, Pearson *et al*. reported that rats immunized with Complete Freund's Adjuvant (CFA) containing *Mycobacterium tuberculosis* (*M. tuberculosis*: MT) developed a form of arthritis termed Adjuvant-Induced Arthritis (AIA) (1). Since this discovery, AIA has been widely used as a model for rheumatoid arthritis (RA). AIA has also been induced using other strains of bacteria, including *M. butyricum* and *Staphylococcus epidermidis* (2, 3). Kohashi *et al*. identified peptidoglycans (PGs), a key component of the bacterial cell membrane, as a common arthritogenic factor among these bacterial cell walls, and muramyl dipeptide (MDP) as the minimum arthritogenic structure of PG's derivatives (4).

AIA can be induced in naïve rats by introducing T-cells (not serum antibodies) from arthritic rats (5). The arthritogenic T-cells recognize an epitope on the 65kDa mycobacterial heat shock protein (HSP) between amino acid residues 180 and 188, which share a similar structure to the link-peptide of proteoglycans found in connective tissues. This indicates that the T-cells respond to self-components and contribute to the induction of arthritis (6,7).

NOTE: While AIA has traditionally been used as a model for RA, AIA differs significantly from RA in its histological and immunological features. AIA should therefore be considered a model for reactive arthritis rather than RA. AIA can be used for studying basic mechanisms of how external triggers may lead to undesired self-recognition (8).

A. Animal Vendors

Even within the same strain, there can be differences in the genetic background and bacterial flora among rats from different vendors. These differences affect how the rats will respond to various reagents, thus impacting experimental results. Chondrex, Inc. recommends performing a pilot study with a defined protocol to test animals from different vendors before proceeding with a large experiment.

B. Housing Conditions

Environmental factors are also important for inducing AIA. For example, Fisher 344 (F344) rats in germ-free conditions are susceptible to AIA and develop severe arthritis compared to their specific pathogen free (SPF) and conventionally housed counterparts (9). F344 rats in conventional housing can still develop AIA, but severity and incidence can be as low as 20%.

C. Rat Strains and Age

Lewis, Sprague-Dawley (SD), Wistar, and Brown Norway (BN) strains are known as high responders to AIA. In contrast, Buffalo (BUF), F344, and the diabetic-resistant subline of diabetic BB (DR BB) rats are medium to low responders to AIA (10,11). The susceptibility of a strain varies by vendor, the dose and species of mycobacteria, and the type of oil used to make the suspension. Six to 12-week-old rats are recommended. Young rats (1 to 7 days old) are not susceptible and old animals (≥9 months) are relatively resistant.

NOTE: Dark agouti (DA) rats are extremely susceptible to AIA, collagen-induced arthritis (CIA), as well as oil induced-arthritis (OIA) which is triggered by the injection of Incomplete Freund's Adjuvant (IFA) or pristane alone. The pathological mechanisms present in this strain may differ from those of "authentic" AIA reported in other strains. Thus, the use of DA rats to study AIA is complex and requires extra consideration.

Table 1 - Rat Strains Commonly used for AIA

High Responder Strains	Lewis
	Sprague-Dawley (SD)
	Wistar
	Brown Norway (BN)
Medium to Low Responder Strains	Buffalo (BUF)
	Fisher 344 (F344)
	Diabetic-resistant Subline of Diabetic BB (DR BB)
Resistant Strain	Wistar Furth (WF)

D. Adjuvant

1. Type of Oil

IFA (Cat # 7002) is widely used as a vehicle, but biodegradable plant oils such as olive oil can also be used (12). It was reported that squalane is most effective for inducing arthritis in low-responding F344 rats (10). In practice, mineral oil, squalane, and liquid paraffin are equally effective for inducing arthritis in a variety of AIA-susceptible rat strains.

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2. Heat-killed Mycobacteria

Various strains of heat killed mycobacteria, such as MT and isolated PGs and MDP can be used as antigens for inducing AIA. The particle size of MT powder is a very important factor for successfully inducing AIA as small particles are more arthritogenic than large particles or aggregates (3). Chondrex, Inc's MT receives extreme grinding to produce very fine particles. To effectively induce arthritis, a suspension containing at least 5 mg/ml of heat-killed mycobacteria is required. Heat-killed MT (10 mg/ml) suspended in IFA (Cat # 7027) has been widely used to induce AIA.

NOTE: Commercial preparations of Complete Freund's Adjuvant (CFA) containing 1 mg/ml of heat-killed MT are seldom effective in inducing AIA because of the low concentration of MT.

Catalog #	Description
7002	Incomplete Freund's Adjuvant, 5 ml
7027	Complete Freund's Adjuvant, 5 ml x 10 mg/ml
7024	Complete Freund's Adjuvant, 5 ml x 20 mg/ml

E. Administration Routes

AIA can be induced in susceptible strains by a single subcutaneous injection of adjuvant into a footpad or at the base of the tail. To successfully induce arthritis in low responder rats, the adjuvant must enter the lymphatic system to trigger immunity in the draining lymph nodes. It was reported that injecting adjuvant into the intra-inguinal lymph node was much more effective for inducing arthritis in low responding F344 rats than the simple subcutaneous injection in footpads (10).

Before each injection, it is vital to re-suspend the adjuvant as the mycobacterial particles will settle. Failure to re-suspend the adjuvant could lead to inconsistent distribution of mycobacterial particles, leading to poor reproducibility.

NOTE: It is not necessary to use an emulsified adjuvant to induce AIA. However, to avoid the injection of inconsistent amounts of MT, CFA can be emulsified with an equal volume of saline or buffer solution and used for injection. This method for inducing AIA has been reported in F344 rats injected with mycobacterial peptidoglycans or MDP (9). For more information about emulsion preparation, please refer to Chondrex, Inc.'s Rat Collagen-Induced Arthritis protocol.

PROTOCOLS TO INDUCE AIA

A. Adjuvant Preparation

Chondrex, Inc. provides CFA containing 10 and 20 mg/ml of specially prepared heat-killed MT (Cat # 7027 and 7024). Suspend CFA, then draw suspension into a 1-ml glass syringe attached to a 25-G needle. Chondrex, Inc. recommends resuspending CFA in the glass syringe by rolling the syringe between the hands for every rat to be injected.

B. Injection

Choose an injection site at either the footpad or the base of the tail, depending on the purpose of the study.

Footpad

Subcutaneously inject 0.05 ml of CFA containing 10 mg/ml of heat-killed MT (Cat # 7027) into the footpad of a rear paw. The needle should be inserted just under the skin of the footpad pointing toward the ankle: this maximizes delivery of the adjuvant to the draining popliteal lymph nodes. Severe and acute inflammation is observed within 30 minutes of injection, peaks within 3 to 4 days, and often persists for 20 to 25 days. The injected foot should not be included in an arthritis severity scoring protocol because swelling always occurs in the injected foot even when arthritis is absent in other limbs.

Secondary arthritis typically appears in the non-injected paws on day 12 to 14 after the injection and peaks within 2-3 days after the onset. Active joint inflammation usually lasts 20 to 25 days; however, in some rats, inflammation can be replaced by slow, progressive bony hypertrophy resulting from periosteal reactions. Footpad injection of adjuvants and other substances are often restricted at many institutions. Regulations regarding the ethical use of animals should be reviewed and approval obtained from the appropriate committee(s) before any studies are conducted.

Base of the tail

Subcutaneously inject 0.1 ml of CFA containing 10 mg/ml of heat-killed MT (Cat # 7027) at the base of the tail (Figure 1). This route is advantageous because it allows all four paws to be scored for arthritis. As seen with footpad injections, severe arthritis typically appears in one or all paws between on day 12 to 14 after the injection and often persists through days 20 to 25.

NOTE: The volume of adjuvant can be reduced to 50% using CFA containing 20 mg/ml of heat-killed MT (Cat # 7024). This option applies to all routes of injection.

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Figure 1. Subcutaneous Injection at the Base of the Tail

EVALUATING ARTHRITIS

A. Scoring

The severity of arthritis can be scored by visual inspection. On days 10 to 25, evaluate disease development by daily macroscopic inspection. When tail base injections are used, all four paws are scored on a scale of 0 to 4 where 0= normal, 1=the mildest arthritis, and 4=the most severe arthritis. The maximum arthritis score using the tail base injection method is 16 (scoring all four paws), while the maximum score for the footpad injection method is 12 (only three paws are scored) (Table 2). In addition to daily scores, the maximum arthritis index (MAI) can be calculated at the end of the study for each rat by adding up the greatest score recorded for each paw. The latter system best reflects the cumulative severity of arthritis, particularly when the onset and resolution of arthritis in the fore- and hind-paws occurs asynchronously (11).

Table 2 - Qualitative scoring system used to assess severity of paw inflammation.

Score	Condition
0	Normal
1	Mild, but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits
2	Moderate redness and swelling of ankle or wrist
3	Severe redness and swelling of the entire paw including digits
4	Maximally inflamed limb with involvement of multiple joints

B. Paw Thickness Assessment

Along with scoring arthritis, foot swelling can also be objectively determined by measuring paw thickness using a caliper, such as the Mitsutoyo loop-handle dial thickness gauge.

C. Paw Volume Assessment

If using a protocol that injects at the base of the tail, the hind paw volume can be measured at select time points. Place a beaker containing a 10% soap solution of a known density on a top-loading balance and tare the balance to zero. Light anesthesia may facilitate this process. Immerse the limb in the solution to the level of the anatomical hair line. After retracting the paw, the container is weighed, subtracted from the start weight, and corrected for fluid density (13). Repeat the measurement for the other paws.

D. Local Hyperthermia Assessment

Local hyperthermia is a very accurate indicator of the severity of an acute inflammatory reaction (14, 15) and is highly recommended because of its simplicity and sensitivity. In performing this measurement, the surface temperature of each paw can be measured by a contact-type thermometer (Thermomex TH-10, Natsume Seisakusho, Tokyo).

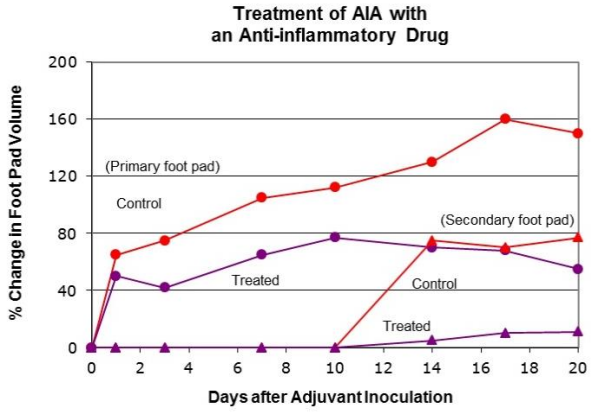

Figure 2 - Evaluating Anti-inflammatory Agents in AIA

AIA was induced in SD rats by injecting 0.05 ml of heat-killed *M. butyricum* suspended in paraffin oil (10 mg/ml) in the right hind footpad on day 0. An anti-inflammatory drug (E-5110: 3 mg/kg) was administered orally every day from day 0 to 20. The effects of the drug were determined by the primary inflammatory reaction at the right hind paw (left) from day 1 to 20, and the secondary inflammatory reaction at the left hind paw (right) from day 14 to 20. Red: Control rats. Purple: Rats treated with an anti-inflammatory drug.

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