A Protocol for Successfully Inducing Collagen-Induced Arthritis (CIA) in Rats

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BACKGROUND

Collagen-induced arthritis (CIA) was originally established in Wistar (outbred), Sprague-Dawley (outbred), and Wistar-Lewis (inbred) rats immunized with type II collagen and later expanded to mice and non-human primates (1-3). These CIA models have been widely used as a model of human rheumatoid arthritis (RA), as they share both immunological and pathological features of RA. However, there are several differences among species with respect to the CIA model. Susceptibility to CIA is linked to the MHC types. For example, mouse CIA is highly restricted by the MHC type (4), but rat CIA is less restricted by the MHC type (5). Thus, a variety of rat strains with different MHC types develop arthritis, although the incidence and severity of arthritis, as well as antibody and T-cell epitope specificity varies among individual strains (6). Although the rat CIA model is highly reproducible, certain considerations must be considered to successfully induce arthritis with sufficient incidence and severity. The following are considered the most important factors to successfully induce rat CIA. These factors must be studied beforehand, especially for first time users of this model.

A. Animal Vendors

From vendor to vendor and even within the same strain, the genetic background and bacteria flora will vary among rats. These differences affect how the rats will respond to various reagents, thus impacting experimental results (7, 8). Chondrex, Inc. recommends testing animals from different vendors using a defined protocol before proceeding with a full-scale experiment.

B. Housing Condition & Diet

Chondrex, Inc. recommends housing animals in Specific Pathogen Free (SPF) conditions rather than conventional conditions to avoid variations within experiments caused by bacterial and viral infections. For example, Lewis rats are highly responsive to CIA and the incidence and severity of arthritis is very high. However, the susceptibility to CIA in this strain varies notably depending upon the age, the breeder, and the inoculated intestinal flora (Terato, *et al*., unpublished observation). It is well known that diet affects the incidence and severity of CIA in mice (9). However, this has not been extensively studied in rats. Diet may not be as important for rats as they respond to type II collagen quicker and develop arthritis within a shorter time period compared to mice. However, benefit may be gained by also feeding a high fat diet as is recommended for mice.

C. Rat Age & Strains

Rats should be at least 7-8 weeks old with a mature immune system. The following strains of rats are known to be highly susceptible to CIA: Sprague-Dawley (SD)(RT1f), Wistar (RT1u), BioBreeding/Diabetes-resistant (BB/DR)(RT1u), Wistar Furth (RT1u), Louvain (LOU) (RT1u), Osborne-Mendal (OM) (RT1u), and Lewis (LEW) (RT1l) rats (1,5,10) (Table 1).

BB rats are the best strain to use for CIA (Biomedical Research Models, Inc., Massachusetts, USA). Dark Agouti (DA) rats are highly susceptible to CIA and less susceptible to oil-induced arthritis (mineral oil: IFA, alone) (11). Therefore, CIA in DA rats may simultaneously involve two different pathogenic mechanisms. Thus, DA rats are not recommended for the CIA model.

NOTE 1: Rat susceptibility to CIA is linked to the MHC RT1 locus and varies with the species of type II collagen used for immunization. In general, porcine type II collagen is the most arthritogenic species of type II collagen followed by bovine, with chicken being the least arthritogenic (5).

NOTE 2: In contrast to mice, rats immunized with autologous rat type II collagen will develop arthritis (1).

NOTE 3: Rat antibodies to type II collagen are highly specific to the conformation of the collagen molecule. Denatured collagen and individual CB peptide fragments of type II collagen are not arthritogenic in rats while they are in mice and monkeys (12-13).

Table 1 - Rat strains used for CIA

Strains	MHC RT1 Haplotype	References	Notes
Sprague-Dawley (SD)	f	1	
Wistar	u	1	
Lewis (LEW)	l	1,5	MDP can be effective in LEW rats for higher incidence and severity of CIA (10)
BioBreeding (BB)/Diabetes-resistant (DR	u	9	
Wistar Furth	u	5	
Louvain (LOU)	u	5	
Osborne-Mendal (OM)	u	5	
Dark Agouti (DA)	av1	5	Susceptible to oil-induced arthritis

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D. Adjuvant

As rats are generally susceptible to adjuvant-induced arthritis (AIA), Incomplete Freund's Adjuvant (IFA), Catalog # 7002, must be used.

Catalog #	Description
7002	Incomplete Freund's Adjuvant, 5 ml

E. Collagen

Highly purified type II collagen prepared under a defined protocol should be used as deglycosylation of collagen will affect the arthritogenicity (14). Moreover, the failure to remove minor contaminants such as pepsin likely yields false positive reactions in a T-cell stimulation assay. Lyophilized collagen is very stable if properly stored at -20°C in the dark. Collagen should be dissolved at 2-4 mg/ml in 0.05M acetic acid (Cat # 9075) by gently stirring overnight at 4°C. Collagen solutions can be kept at 4°C for one week but should then be kept at -20°C thereafter. Chondrex, Inc. offers a complete line of immunization grade type II collagen:

Catalog #	Description
20011	Chick type II collagen, 10 mg
20012	Chick type II collagen, 5 ml x 2 mg/ml
20021	Bovine type II collagen, 10 mg
20022	Bovine type II collagen, 5 ml x 2 mg/ml
20032	Porcine type II collagen, 5 ml x 2 mg/ml
20041	Rat type II collagen, 5 mg
20042	Rat type II collagen, 2.5 ml x 2 mg/ml
9075	Collagen Solubilizing Buffer (0.05M Acetic Acid), 5 ml

PROTOCOL TO INDUCE ARTHRITIS

A. Preparing the Emulsion

The quality of the emulsion for immunization is critical for inducing arthritis with high incidence. Emulsions can be made using various methods. However, syringe-syringe or sonication methods are not recommended. These methods yield emulsions that are not stable enough to effectively induce arthritis. In addition, sonication cleaves collagen into fragments which will be denatured at body temperature.

An electric homogenizer is highly recommended for preparing an emulsion:

1. Use a homogenizer (Figure 1) with a small blade (diameter of 5 mm or less) to emulsify the IFA (Catalog # 7002) with the collagen solution (Figure 2a). Seal the tip of the syringe with a 3-way stopcock. Next, clamp the syringe to a ring stand and place it in an ice water bath (Figure 3) to keep the emulsion cool during mixing, as heat will denature the collagen and fail to induce arthritis.

Figure 1 – Homogenizer

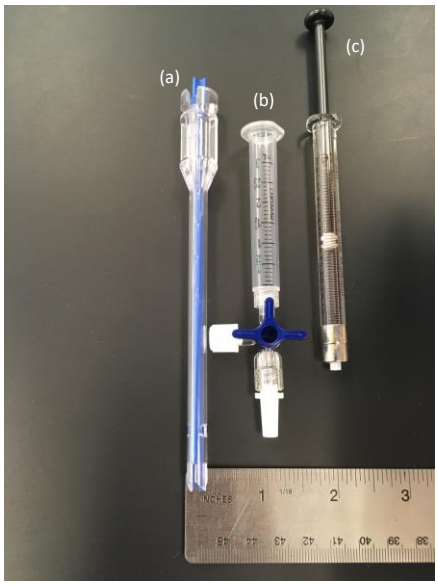

Figure 2 - Homogenizing blade - 0.5 cm diameter (a), Syringe with a 3-way stopcock (b), Hamilton glass syringe - 1 ml (c).

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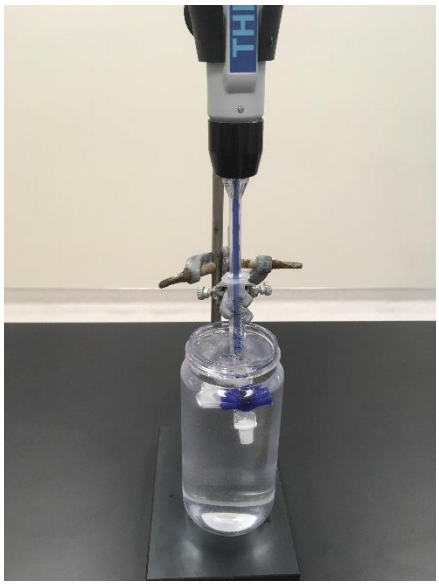

Figure 3 - A syringe clamped to a stand and placed in an ice water bath.

Note: Seal the needle end with a 3-way stopcock.

2. Add one volume (maximum: ¼ of syringe volume) of IFA to the syringe sealed with a 3-way stopcock. Then slowly add an equal volume of collagen solution (2 mg/ml in 0.05M acetic acid) dropwise while mixing at low speed (1000-3000 rpm).

   NOTE: To ensure a high-quality emulsion, the maximum emulsion volume should be half of the syringe volume. If more is needed, make several batches.

3. Continue mixing the emulsion at maximum speed (approximately 10,000 - 30,000 rpm) for 2 minutes. Cool down the emulsion by keeping the syringe in the ice water bath for 5 minutes. Repeat mixing and cooling 2-3 times. For larger volumes (2-5 ml), we suggest moving the blade throughout the emulsion while mixing for better uniformity.

4. Swap out the stopcock for a needle and test the stability of the emulsion by adding one drop of emulsion into a beaker of water. If the emulsion is stable, the drop will remain as a solid clump which does not dissipate on the water's surface.

   NOTE: If the emulsion dissipates onto the water surface then the emulsion is not stable. Add a few drops of adjuvant, mix again, and retest.

5. Transfer the emulsion to a 1 ml Hamilton glass syringe (Figure 2c). Injecting an accurate volume of emulsion is difficult with a plastic syringe.

   NOTE 1: Remove air bubbles from the emulsion by forcefully swinging your arm towards the floor, with the Hamilton syringe in hand (plunger side down).

   NOTE 2: Chondrex, Inc. recommends injecting the collagen emulsion within an hour of preparation. Keep the emulsion cool at 4°C until use.

B. Injection Site

Place a 25 or 27 gauge x 5/8" needle on the Hamilton syringe. Before each injection, wipe the needle to prevent leakage of the emulsion. Insert the needle bevel side up and parallel to the tail 2 cm from the base of the tail until the needle tip is 0.5 cm from the base. The entire needle should be subcutaneous. Inject 0.2 ml (200 µg collagen/rat) of the emulsion subcutaneously at the base of the tail (Figure 4). For a booster injection, insert the needle at 3 cm from the base of the tail until the tip reaches 1.5 cm from the base. The booster injection should be administered at a different location from the initial injection.

Note: Chondrex, Inc. does not recommend subcutaneous injections in the back nor intraperitoneal (IP) injections, as emulsions cause severe inflammatory reactions in the peritoneal and thoracic cavities.

Figure 4 – Subcutaneous Immunization of Emulsion.

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C. Immunization Schedule

There are several ways to induce arthritis with high incidence and high severity depending on the rat strain and the experimental purpose.

1. **Inducing arthritis by a single immunization without booster injection**:
   Follow the protocols for the preparation of emulsion (collagen-IFA) and injection site as stated above, and arthritis will develop 2-3 weeks after immunization depending on the strain. The incidence of arthritis should be 80-100% in high responder strains. The severity of arthritis should reach a score of 10-12 (maximum score 16) in highly susceptible strains such as BB rats.

2. **Inducing arthritis with a booster injection**:
   To ensure high incidence and severity of arthritis, a booster injection can be given on day 7 after the initial immunization. Prepare the collagen-IFA emulsion as described above and administer 0.1 ml of the emulsion subcutaneously in the tail. Rapid onset of severe arthritis is expected.

3. **Efficient induction of arthritis using synthetic muramyl dipeptide as an adjuvant**:
   As Complete Freund's Adjuvant containing *M. tuberculosis* cannot be used to immunize rats with type II collagen, N-acetylmuramyl-L-alanyl-D-isoglutamine hydrate (MDP) can be used as an alternative adjuvant. Dissolve MDP at 4-8 mg/ml in distilled water. The collagen concentration should be 4 mg/ml in 0.05M acetic acid. Mix an equal volume of MDP and collagen solution before use. Make an emulsion by mixing the MDP-collagen solution with an equal volume of IFA as described above. Inject 0.2 ml of the emulsion subcutaneously in the tail. Arthritis incidence, severity, and consistency (from experiment to experiment) using this method may be greatly increased compared to the original protocol of using IFA alone (15).

   Note: MDP alone induces arthritis at higher doses (~0.8 mg per rat). Thus, it is recommended to optimize the dose in individual strains of rats before using this protocol.

D. Onset of Arthritis

Clinically apparent arthritis with swollen joints appears 2-3 weeks after the first immunization. The onset of arthritis in BB rats tends to be earlier than other strains of rats, around 12-14 days. If immunization is effective, 100% of rats will develop arthritis in around 3 weeks.

EVALUATING ARTHRITIS

A. Scoring

Disease can be assessed by a qualitative clinical score or by determining paw thickness using a thickness gauge, such as a Mitutoyo loop handle dial thickness gauge with a round disc. These methods are applicable for all arthritis models including classic CIA, adjuvant-induced arthritis, cotton-induced arthritis (16), and other inflammatory models. Chondrex, Inc. provides a scoring system (Table 2).

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 Volume Assessment:

At chosen time-points, the hind paw volume can be measured. 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 (17). Repeat the measurement for the other paws.

C. Serum Analysis:

High IgG autoantibody levels to type II collagen are important for inducing arthritis (8,12). More specifically, high levels of anti-type II collagen IgG2a and/or IgG2b subtype antibodies are required to activate complement, an essential step for inducing arthritis. Chondrex, Inc. provides rat Anti-Collagen IgG and IgG subtype antibody ELISA kits to analyze the antibody levels. Please visit www.chondrex.com for more information.

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