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

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BACKGROUND

Collagen-induced arthritis (CIA) in mice shares immunological and pathological features with human rheumatoid arthritis (RA). The CIA model is ideal to study the pathogenesis of RA and to test therapeutics (1-3). Although the model is highly reproducible, certain considerations must be taken into account to successfully induce arthritis with sufficient incidence and severity. Therefore, a pilot study is recommended for first time users of this animal model.

A. Animal Vendors

From vendor to vendor and even within the same strain, the genetic background and bacteria flora will vary among mice. These differences affect how the mice will respond to various reagents, thus impacting experimental results (4). 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 For example, mice infected with mouse hepatitis virus (MHV) will not develop CIA (unpublished observation). The incidence and severity of arthritis varies in mice fed with different commercially available rodent chows. The highest disease incidence has been observed in mice fed a high fat diet designed for breeders (Purina Mouse Chow 5015) (5).

C. Mouse Age & Strains

Mice should be at least 7-8 weeks old with a mature immune system. Aged mice may exhibit poor incidence and severity. Chondrex, Inc recommends consistently using the same age for better reproducibility in repeat studies. Susceptibility to CIA is linked to MHC-class II molecules which respond to individual species of type II collagen used for immunization (6). DBA/1 (H-2^q) and B10.RIII (H-2^r) mice are highly susceptible to CIA. DBA/1 mice respond to chick, bovine, and porcine type II collagen. B10.RIII mice respond to bovine and porcine type II collagen but respond poorly to chick type II collagen. DBA/1 (H-2^q) and B10.RIII (H-2^r) mice respond poorly to mouse type II collagen. Even after extensive immunization with mouse type II collagen, CIA incidence is still very low (approximately 10%) (7).

On the other hand, some CIA resistant mouse strains can produce arthritogenic antibodies, suggesting that CIA is not only restricted by MHC types. For example, INF-γ or IL-10 knockout CIA resistant C57BL/6, 129/Sv (H-2^b), and Balb/c (H-2^d) mice can produce arthritogenic autoantibodies and develop arthritis. This indicates that susceptibility to arthritis is also highly regulated by cytokines (8).

A list of mouse strains commonly used for CIA and Collagen Antibody-Induced Arthritis (CAIA) are shown in Table 1.

Table 1 - Mouse strains commonly used for CIA and CAIA

Mouse Strain	H-2 Type	CIA Susceptibility	Ref #	CAIA Susceptibility	Ref #	Note
DBA/1	q	High	2, 5, 6	High	13, 21	INFγ high
B10.Q	q	High	6	(High)		
B10.G	q	High	6	(High)		
NFR/N	q	High	38	(High)		
SWR	q	Resistant	17	Resistant		C5 deficient
B10.RIII	r	High	6	High	13	Low response: chick and human type II
B10	b	Low	10	(High)		* Need alternative immunization
C57BL/6	b	Low	10	Moderate - High	9, 18, 30	LPS low responder – * Need alternative immunization
C57BL/6 beige	b	Resistant	20	Resistant		PMN mutation
C57BL/6 x 129/Sv	b	Low	10	Moderate - High	30, 31	* Need alternative immunization
129/Sv	b	Resistant	10	High	27	
B10.D2/nSn	d	Resistant	20	High	20	
B10.D2/oSn	d	Resistant	20	Resistant	20	C5 deficient
Balb/c	d	Resistant		High	13	
Balb/c nu/nu	d	Resistant		Resistant	28	B & T cell deficient
C3H/He	k	Low	38	(Low)		
B10.S	s	Resistant	5	?		
SJL/1	s	Moderate	2	(High)		
C.B-17 scid/scid		Resistant		High	18	B & T cell deficient

Parenthesis-assumed, but not tested
*Develops arthritis by alternative immunization with CFA containing high concentrations of *M. tuberculosis*.

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D. Adjuvant

Complete Freund's Adjuvant (CFA), consisting of high-quality *M. tuberculosis*, is essential to induce severe arthritis in mice because it induces a strong immune response. Unlike rats, mice will not develop arthritis by immunizing with type II collagen emulsified with Incomplete Freund's Adjuvant (IFA). A strong antibody response as well as the correct antibody subtype is critical for inducing arthritis in mice. Antibody production depends on the concentration of *M. tuberculosis* in CFA, and sufficient anti-collagen IgG2a and IgG2b subtype antibody levels are necessary to activate complement, an essential step for inducing arthritis (9). In fact, Campbell, *et al*. reported that CFA containing 5 mg/ml of *M. tuberculosis* successfully induced arthritis with high incidence (50-70%) in CIA resistant mouse strains, such as C57BL/6, B10, and 129/Sv mice (H-2^b) (10). However, because high concentrations of *M. tuberculosis* induce severe inflammation, please contact your institution's animal committee for guidance on choosing the appropriate CFA. The following is a list of adjuvants provided by Chondrex, Inc.

Catalog #	Description
7002	Incomplete Freund's Adjuvant, 5 ml
7008	Complete Freund's Adjuvant, 5 ml x 1 mg/ml
7009	Complete Freund's Adjuvant, 5 ml x 2 mg/ml
7015	Complete Freund's Adjuvant, 5 ml x 3 mg/ml
7001	Complete Freund's Adjuvant, 5 ml x 4 mg/ml
7023	Complete Freund's Adjuvant, 5 ml x 5 mg/ml

E. Collagen

Native, highly purified type II collagen prepared under a defined protocol should be used as deglycosylation of collagen will affect the arthritogenicity (11). Moreover, the failure to remove minor contaminants such as pepsin likely yields false positive reactions in T-cell proliferation assays (12). 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 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 for the CIA model depending on the mouse strain (please see Table 1 for more information). For example, DBA/1 mice strongly respond to chick or bovine type II collagen, whereas C57BL/6 mice only respond to chick 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
20031	Porcine type II collagen, 10 mg
20032	Porcine type II collagen, 5 ml x 2 mg/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 CFA (IFA for booster injection) 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 to keep the emulsion cool during mixing, as heat will denature the collagen which will then fail to induce arthritis (Figure 3).

Figure 1 – Homogenizer

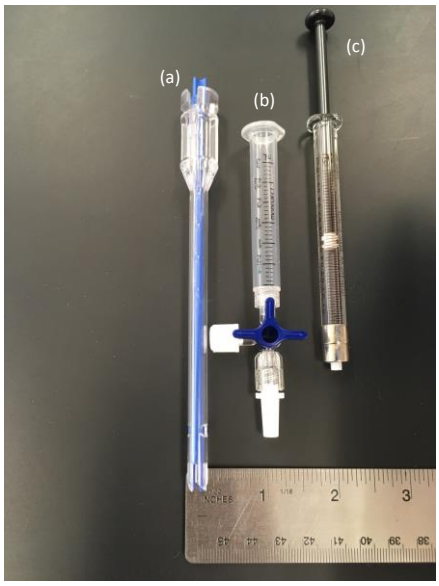

Figure 2 - Homogenizing blade - 0.5 cm diameter (a), Syringe with a 3-way stopcock (b), Hamilton glass syringe - 1 ml (c).

Figure 3 - A syringe sealed with a 3-way stopcock, clamped to a stand, and placed in an ice water bath.

2. Add one volume (maximum: 25% of the syringe volume) of CFA (IFA for booster injection) to the end of the syringe sealed with the 3-way stopcock. Then gradually 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 no more than half of the syringe volume (50%). 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. Replace the stopcock with 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. If the emulsion dissipates onto the water surface, then the emulsion is not stable. Add a few drops of adjuvant, mix again, and retest.

Figure 4 - An intact, stable emulsion on the water's surface

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 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 0.1 ml (100 µg collagen/mouse) 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.

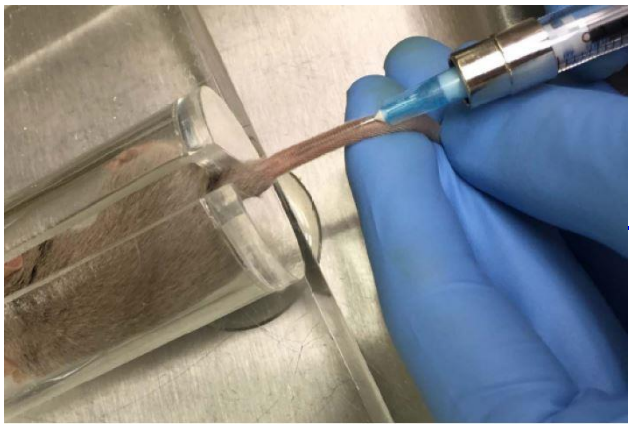

Figure 5 - Subcutaneous Immunization of Emulsion.

C. Immunization Schedule

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

1. **Inducing arthritis by a single immunization without booster injection in HIGH RESPONDER strains**:

   Inject the emulsion of collagen and CFA containing a final concentration of 2 mg/ml of *M. tuberculosis* in mice. Arthritis will develop on days 28-35 after immunization in CIA high responder strains, such as DBA/1 (H-2^q) and B10.RIII (H-2^r) mice. The incidence of arthritis is usually 90-100% on days 42-56. The severity of arthritis can be high and reach a score of 10-12 (maximum score 16).

   NOTE: Inflammation at the injection site is generally severe because of the high concentration of *M. tuberculosis*. Thus, some facilities may not accept this protocol. In this case, use one of the following protocols (2) or (4).

2. **Inducing arthritis with a booster injection in HIGH RESPONDER strains**:

   Inject the collagen and CFA emulsion containing a final concentration of 0.5 mg/ml of *M. tuberculosis*. Administer a booster injection with a collagen and IFA emulsion on day 21. The booster injection should be administered at a different location than the first injection site. Arthritis will develop on days 28-35 after the first immunization. The incidence of arthritis is around 80-100% and severity of arthritis can reach scores of 8-12 (maximum score 16) on days 42-56.

3. **Inducing arthritis with an alternative immunization protocol with high *M. tuberculosis* content adjuvant in LOW RESPONDER strains**:

   CIA can be induced in several CIA low responder mouse strains such as B10 (H-2^b), C57BL/6 (H-2^b), and C57BL/6x129/Sv (H-2^b). Inject the collagen and CFA emulsion containing a final concentration of 2.5 mg/ml of *M. tuberculosis*. Administer a booster injection with an emulsion of collagen and CFA containing a final concentration of 2.5 mg/ml of *M. tuberculosis* on day 21. Arthritis will develop on days 28-35 after the first immunization. The maximum incidence of arthritis in these mice reaches approximately 50-70% on days 42-56 (10).

   NOTE: The inflammatory reaction at the injection site might be very severe, thus some animal committees may not accept this protocol. An alternative mouse arthritis model with no inflammation at the injection site as well as a dramatically shorter experimental period is the collagen antibody-induced arthritis (CAIA) model. Chondrex, Inc.'s anti-type II collagen monoclonal antibody cocktail (Arthrogen-CIA®) and LPS will induce arthritis in these CIA resistant mouse strains. Please visit www.chondrex.com for more information.

4. **Synchronizing onset of arthritis by LPS injection**:

   LPS has a synergistic effect in triggering arthritis with sub-arthritogenic doses of autoantibodies to type II collagen (13). Furthermore, severity and incidence in CIA can be increased by administering LPS (a B-cell mitogen), *Mycoplasma arthritidis* (a T cell mitogen) (MAM), and Staphylococcal enterotoxin B (SEB) (14-16). These bacterial toxins can be used not only to trigger and enhance arthritis, but also to synchronize the onset of arthritis.

   For this protocol, inject the collagen and CFA emulsion containing a final concentration of 0.5 mg/ml of *M. tuberculosis* according to protocol (2). Inject LPS (25-50 µg in saline) intraperitoneally on day 25-28 or 3-5 days before the desired onset of arthritis. Arthritis will develop within 24-48 hours in 90-100% of mice.

   NOTE: Mice immunized with CFA develop severe immune suppression for 2-4 weeks following the first immunization. Therefore, some mice will be highly susceptible to LPS injection (50 µg). As previously mentioned, (see Animal Vendors), Chondrex, Inc. suggests testing animals from different vendors before proceeding with a full-scale experiment.

D. Onset of Arthritis

Clinically apparent arthritis with swollen joints appears 3-5 weeks with effective immunization. Onset and incidence of CIA depends on mouse strains and protocols.

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 CIA, adjuvant-induced arthritis, CAIA, and other inflammatory models. Chondrex, Inc. provides a scoring system (Table 2) and a supplemental flyer (please visit www.chondrex.com).

NOTE: Mouse paw volume cannot be determined by a plethysmograph as used for rat paw volume measurement because the mouse paw is too small.

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. Serum Analysis

High IgG autoantibody levels to mouse type II collagen are important for inducing arthritis (10, 17). More specifically, high levels of anti-type II collagen IgG2a and IgG2b subtype antibodies are required to activate complement, an essential step for inducing arthritis. Chondrex, Inc. provides mouse 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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