

Protocol for the Successful Induction of Collagen-Induced Arthritis (CIA) in Rats

Collagen-induced arthritis (CIA) was first developed using Wistar (outbred), Sprague-Dawley (outbred) and Wistar-Lewis (inbred) rats by immunization with type II collagen (1). This model was expanded to mice (2) and non-human primates (3) and has been widely used as a model of human rheumatoid arthritis (RA), since the CIA model shares both immunological and pathological features with RA. However, there are several differences among species with respect to the CIA model. Susceptibility to CIA links to the MHC in mice (4), thus only two strains of mice, DBA/1 (H-2^d) and B10.RIII (H-2^k), are susceptible to CIA. While susceptibility to CIA in rats is also linked to the MHC (5), it is much more broad compared to mice. Thus, a variety of 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).

The rat CIA model is highly reproducible. However, there are certain considerations that must be noted and are required for inducing arthritis with a high incidence and severity. The following are considered to be the most important factors to successfully induce arthritis in rats. It is imperative that these factors are studied beforehand, especially for first time users of this model.

Animal Care and Diet

Animals should be healthy and young (7-8 weeks old) and maintained under SPF conditions. In general, the intestinal bacteria flora, regardless of whether it is pathogenic or non-pathogenic, affects the host's immune responses to antigens significantly. Germ free rats are highly susceptible to adjuvant-induced arthritis (AIA) followed by SPF and conventional rats being less susceptible (7). Also, the incidence and severity of CIA, even in the same strain, can differ significantly depending on the breeder.

It is well known that diet affects the incidence and severity of CIA in mice (8). However, this has not been extensively studied in rats. Diet may not be as important for rats since they respond to type II collagen quicker and develop arthritis within a shorter period of time compared to mice. However, benefit may be gained by also feeding a high fat diet as is recommended for mice.

Strains of Rats

The immune response to type II collagen and subsequent development of arthritis in rats is linked to the MHC RT1 locus and varies with the species of type II collagen used for immunization. However, compared to mice, a variety of rat strains are susceptible to CIA and develop arthritis to a limited extent. In general, porcine type II collagen is the most potent arthritogenic species of type II collagen followed by bovine, with chicken being the least arthritogenic (5). The following strains of rats are known to be highly susceptible to CIA: BB/DR (RT1^u) (9), Wistar Furth (RT1^u), LOU (RT1^u), OM (RT1^u), and LEW (RT11) rats (5).

The quality of the animals is also very important. In this regard, intestinal flora may be one of the most important factors affecting the immune response to type II collagen and subsequent development of arthritis. 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 intestinal flora inoculated when they are converted to SPF (Terato, et al., unpublished observation). MDP can be very effective in Lewis rats to enhance the incidence and severity of CIA (10). However, we recommend testing Lewis rats in a small-scale experiment prior to a large-scale experiment. To our knowledge, BB rats are the best strain to use for CIA (Biomedical Research Models, Inc., Massachusetts, USA).

DA rats are susceptible to CIA, but are also highly susceptible to adjuvant-induced arthritis (AIA) and oil-induced arthritis (mineral oil alone) without adjuvant, thus is not recommended for use in the CIA model (11).

Note 1: In contrast to mice, rats are susceptible to autologous rat type II collagen (1) and develop arthritis as has been observed in monkeys (12).

Note 2: 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 which is contrary to mice (13) and monkeys (12).

Collagen

Highly purified type II collagen prepared under a defined protocol should be used since deglycosylation of collagen will affect the arthritogenicity (14), while 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 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
20031	Porcine type II collagen, 10 mg
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
20051	Human type II collagen, 1 mg
20052	Human type II collagen, 0.5 ml x 2 mg/ml
20061	Mouse type II collagen, 1 mg
20062	Mouse type II collagen, 0.5 ml x 2 mg/ml

Preparation of Emulsion

Since rats are generally susceptible to adjuvant-induced arthritis (AIA), incomplete Freund's adjuvant (IFA), catalog # 7002, must be used.

The quality of the emulsion for immunization is critical for inducing arthritis at a 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 induce arthritis effectively. In addition, sonication cleaves collagen into at least two fragments, which will be easily denatured at body temperature.

An electric homogenizer is highly recommended for use as follows:

- 1) Use a homogenizer (Figure 1) with a small blade (diameter of 5 mm or less) to mix the IFA and collagen solutions (Figure 2a). If the blade cannot reach the bottom of the mixing syringe, it is convenient to use a 5 ml or 10 ml syringe that is cut halfway from the plunger opening (Figure 2b). Clamp the syringe to a ring stand and place it in an ice water bath (Figure 3). This last step is crucial to prevent denaturation of the collagen as it warms during mixing. Denatured collagen will not induce CIA.



Figure 1 - Homogenizer (Virtis)



Figure 2 - (a) Homogenizing blade - 0.5 cm diameter (b) Cut syringe - 10 ml with a 3-way stopcock (c) Hamilton glass syringe - 1 ml



Figure 3 - A 10 ml syringe, which has been cut from the plunger end, 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 = 2.5 ml) of CFA (IFA for booster injection) to the syringe with a 3-way stopcock. Add an equal volume of collagen solution (2 mg/ml in 0.05M acetic acid) drop-wise while mixing at low speed.
- 3) Continue mixing until a stiff emulsion results at maximum speed (approximately 30,000 rpm for 2-3 minutes). Make sure the emulsion is cooled on the ice water bath prior to mixing again. For larger volumes (2-5 ml), we suggest moving the blade throughout the emulsion while mixing for better uniformity.
- 4) 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.

Note: If the emulsion spreads 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 Hamilton glass 1 ml syringe (Figure 2c). Use of plastic syringes are not suggested, since it is difficult to inject an accurate volume of emulsion.

Note 1: Remove air bubbles throughout the emulsion by forcefully shaking your arm towards the floor, with the Hamilton syringe in hand (plunger side down). Otherwise, it is very difficult to inject an accurate volume of emulsion.

Note 2: We recommend injecting the collagen emulsion within an hour of preparation. Keep the emulsion cool at 4°C until use.

Injection Site

Inject 0.2 ml (collagen: 200 µg) of the emulsion subcutaneously at the base of the tail (Figure 4). For example, insert a 25 or 27 gauge x 5/8" needle at 2 cm from the base of the tail until needle tip reaches 0.5 cm from the base. Needle length should be completely subcutaneous and wiped before each injection to prevent leakage of emulsion. The needle should be inserted bevel up and parallel to the tail. If a booster injection is given, insert needle at 3 cm from the base of the tail until needle tip reaches 1.5 cm from the base.

Note 1: Intradermal injection in the back skin is also effective, but requires larger amounts of collagen emulsion (0.5-1 ml).

Note 2: We do not recommend subcutaneous injection on the back and intraperitoneal (IP) injection of collagen emulsified with adjuvant in any case, such as a primary or a booster immunization, because IFA causes severe inflammatory reactions in the peritoneal and thoracic cavities.



Figure 4 - Immunization of emulsion subcutaneously

Immunization Schedule

For inducing arthritis, IgG autoantibody levels to type II collagen and the subtype (for complement activation) are important. There are several ways to induce arthritis with a high incidence depending on the experimental purpose.

a) **Induction of arthritis by a single immunization without booster injection:**

Using the protocols for the preparation of emulsion (collagen-IFA) and injection site above, 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 reaches a score of 10-12 (maximum score 16) in highly susceptible strains such as BB rats.

b) **Induction of arthritis with a booster injection:**

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

c) **Efficient induction of arthritis using synthetic muramyl dipeptide as an adjuvant:**

Complete Freund's adjuvant containing M. tuberculosis cannot be used to immunize rats with type II collagen. However, another adjuvant, N-acetylmuramyl-L-alanyl-D-isoglutamine hydrate (MDP) can be used. 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 (10).

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. Do not use this protocol for DA rats.

Onset of Arthritis

Onset of arthritis in rats is much faster than mice (around 4 weeks) and 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 develop arthritis around 3 weeks.

Arthritis Score

Disease can be assessed by a qualitative clinical score (Table 2) or by determining paw thickness using a thickness gauge, such as a Mitutoyo loop handle dial thickness gauge which consists of a round disc, or paw volume using a plethysmometer (Ugo Basile, Italy or Kent Scientific Corporation, Connecticut, USA) These methods are applicable for all arthritis models including classic CIA, adjuvant-induced arthritis, and cotton-induced arthritis in rats (15).

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

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