

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

Collagen-induced arthritis (CIA) (1-3) shares both immunological and pathological features with human rheumatoid arthritis (RA), therefore it has been used extensively as a model to study the pathogenesis of RA and for testing therapeutics. Although the model is highly reproducible, there are certain considerations that must be noted and are required for inducing arthritis successfully with sufficient incidence and severity.

The following are considered to be the most important factors to successfully induce arthritis in mice. It is imperative that these factors are studied beforehand, especially for first time users of this model. However, as with any biological model, variations exist from institution to institution. Therefore, each investigator must determine their own working protocol.

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. Viral and bacterial infections also affect the immune response to collagen and the subsequent development of arthritis. For example, mice infected with mouse hepatitis virus (MHV) will not develop CIA (unpublished observation).

Diet also affects the incidence and severity of arthritis. CIA varies in mice fed with different commercially available rodent chows. The highest disease incidence has been observed in mice fed with a diet designed for breeders (Purina Mouse Chow 5015) which has a high fat content (4).

Strains of Mice

Susceptibility to CIA is linked to MHC-class II molecules, but also depends on the species of type II collagen used for immunization (5). Two strains of mice are most commonly used, since they are highly susceptible to CIA. They are DBA/1 (H-2^d) mice which respond to chick, bovine, porcine, and human type II collagen, and B10.RIII (H-2^k) mice which respond to bovine and porcine type II collagen only (a poor response to chick and human type II collagen have been noted).

Importantly, both strains of mice respond poorly to mouse type II collagen, and serum antibody levels to mouse type II collagen are less than 1% of arthritogenic levels (Terato, et al. unpublished observation). Therefore, arthritis only develops occasionally (approximately 10%) after extensive immunization with mouse type II collagen (6). A list of mouse strains commonly used for CIA and Collagen Antibody-Induced Arthritis (CAIA) can be found in Table 1.

Table 1 - Mouse strains commonly used for CIA and CAIA

Mouse Strain	H-2 Type	CIA Susceptibility	Reference	CAIA Susceptibility	Ref	Note
DBA/1	q	High	2, 4, 5	High	13, 20	IN γ high
B10.Q	q	High	5	(High)		
B10.G	q	High	5	(High)		
NFR/N	q	High	37	(High)		
SWR	q	Resistant	12	Resistant		C5 deficient
B10.RIII	r	High	5	High	13	Low response: chick and human type II
B10	b	Low	9	(High)		* Need alternative immunization
C57BL/6	b	Low	9	Moderate - High	8, 17, 29	LPS low responder – * Need alternative immunization
C57BL/6 beige	b	Resistant	19	Resistant		PMN mutation
C57BL/6 x 129/Sv	b	Low	9	Moderate - High	29, 30	* Need alternative immunization
129/Sv	b	Resistant	9	High	26	
B10.D2/nSn	d	Resistant	19	High	19	
B10.D2/oSn	d	Resistant	19	Resistant	19	C5 deficient
Balb/c	d	Resistant		High	13	
Balb/c nu/nu	d	Resistant		Resistant	27	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	17	B & T cell deficient

Parenthesis – assumed but have not tested

* – Develops arthritis by alternative immunization with CFA containing high concentration of M. Tuberculosis

On the other hand, CIA resistant mice are not always incapable of producing arthritogenic antibodies. A recent report suggests that the susceptibility to CIA is not only restricted by MHC types, and CIA resistant C57BL/6, 129/Sv (H-2^b), and Balb/c (H-2^d) mice are capable of producing arthritogenic autoantibodies and develop arthritis when INF- γ or IL-10 gene is deleted. This indicates that the susceptibility to arthritis also highly depends on the regulation by a variety of cytokines (7).

Adjuvant

Complete Freund's Adjuvant (CFA) is essential for the induction of arthritis in mice. Unlike rats, mice will not develop arthritis by immunization with type II collagen emulsified with Incomplete Freund's Adjuvant (IFA). Antibody production, including IgG2a antibody which is essential for activating complement and subsequently inducing arthritis, depends on the concentration of *M. tuberculosis* in CFA (8). Recently, Campbell, et al. (9) used CFA, which contains 5 mg/ml of *M. tuberculosis* and successfully induced arthritis at high incidence (50-70%) in CIA resistant strains of mice, such as C57BL/6, B10, and 129/Sv mice (H-2^b). The concentration of CFA depends on the immunization schedule and the recommendation by each institution's animal care and use committee.

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
7027	Complete Freund's Adjuvant, 5 ml x 10 mg/ml
7024	Complete Freund's Adjuvant, 5 ml x 20 mg/ml

Collagen

Highly purified type II collagen prepared under a defined protocol should be used since deglycosylation of collagen will affect the arthritogenicity (10), while the failure to remove minor contaminants such as pepsin likely yields false positive reactions in a T-cell stimulation assay (11). 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

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 CFA (IFA for booster injection) 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.

Note: May require repeat mixing 2-3 times.

- 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 0.1 ml (collagen: 100 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. Subcutaneous injection on the back or intraperitoneally (IP) is not recommended.

Note: We do not recommend IP injection of collagen emulsified with adjuvant in any case such as a primary or a booster immunization, because both CFA and IFA cause severe inflammatory reactions in the peritoneal and thoracic cavities.



Figure 4 - Immunization of emulsion subcutaneously

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Immunization Schedule

For inducing arthritis, IgG autoantibody levels to type II collagen and the subtype (for complement activation) are important (8, 12). 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-CFA containing 4 mg/ml *M. tuberculosis*) and injection site above, arthritis will develop 3.5-4 weeks after immunization in CIA high responder strains, such as DBA/1J (H-2^a) and B10.RIII (H-2^k) mice. The incidence of arthritis should be 90-100% at 6-8 weeks. The severity of arthritis is also high and reaches 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 (b) or (c).

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

Prepare and inject the emulsion as described above for the initial injection using CFA containing 1 mg/ml of *M. tuberculosis*. Administer a booster injection on day 7 or 21. The collagen should be emulsified with IFA instead of CFA for the booster injection. Inject 0.05-0.1 ml of the booster emulsion subcutaneously in the tail, but at a different location from the first injection site. Arthritis will develop 4-4.5 weeks after the first immunization. The incidence of arthritis is around 80-100% and severity of arthritis will reach scores of 8-12 (maximum score 16) at 6-8 weeks, just slightly lower than in protocol (a).

c) **Induction of synchronized arthritis by LPS injection:**

LPS has a synergistic effect in triggering arthritis with sub-arthritis levels of autoantibody to type II collagen (13). Furthermore, classic CIA can be enhanced by an administration of LPS, a B-cell mitogen (14), MAM, a T-cell mitogen produced by *Mycoplasma Arthritis* (15), and SEB, a T-cell mitogen produced by *Staphylococcus aureus* (16). Thus, these bacterial toxins can be used not only to trigger and enhance arthritis, but also to synchronize the onset of arthritis.

Immunize mice with collagen type II-CFA emulsion according to protocol (b). Inject LPS (25-50 µg dissolved in saline) intraperitoneally on day 25-28 or 3-5 days before the desired onset of arthritis in protocol (b). Arthritis will develop within 24-48 hours in 90-100% of mice.

Note: It is assumed that mice immunized with CFA develop severe immune-suppression during 2-4 weeks after the first immunization. Therefore, some mice will be highly susceptible to LPS toxicity and be killed by a single IP injection of LPS (50 µg).

d) **Induction of arthritis by an alternative immunization with high *M. tuberculosis* adjuvant:**

CIA can be induced in several CIA resistant mice such as B10 (H-2^b), C57BL/6 (H-2^b), and C57BL/6x129/Sv (H-2^b) by alternative immunization with type II collagen. Prepare collagen emulsion with CFA containing 5 mg/ml of *M. tuberculosis* and inject 0.1 ml of the emulsion as described above for the initial immunization. However, it is necessary to give a booster injection with collagen emulsified with CFA containing high *M. tuberculosis*. Prepare collagen emulsion with the same CFA instead of IFA, and inject 0.1 ml of the emulsion at the base of tail on day 21. Arthritis will be observed in 4-6 weeks. The maximum incidence of arthritis is approximately 50-70% of these mice at 6-7 weeks (9).

Note: The inflammatory reaction at the injection site might be very severe, thus some facilities may not accept this protocol. As an alternative and better way, we strongly recommend using a combination of antibody (ArthroGen-CIA[®])-LPS for inducing arthritis in these CIA resistant strains of mice as discussed later.

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. These methods are applicable for all arthritis models including classic CIA, antibody-induced, and antibody plus LPS-induced arthritis in mice and other inflammatory models.

Note: 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 of wrist
3	Severe redness and swelling of the entire paw including digits
4	Maximally inflamed limb with involvement of multiple joints

References

- DE Trentham, AS Townes, AH Kang. Autoimmunity to type II collagen: an experimental model of arthritis. *J Exp Med* 146:857-68, 1977.
- JS Courtenay, MJ Dallman, AD Dayan, A Martin, B Mosedale. Immunization against heterologous type II collagen induces arthritis in mice. *Nature* 283:666-668, 1980.
- ES Cathcart, KC Hayes, WA Gonnerman, AA Lazzari, C Franzblau. Experimental arthritis in a nonhuman primate. I. Induction by bovine type II collagen. *Lab Invest* 54:26-31, 1986.
- PH Wooley. Collagen-induced arthritis in the mouse. *Methods Enzymol* 162:361-373, 1988.
- PH Wooley, et al. Type II collagen-induced arthritis in mice. IV. Variations in immunogenetic regulation provide evidence for multiple arthritogenic epitopes on the collagen molecule. *J Immunol* 135:2443-2451, 1985.
- R Holmdahl, L Jansson, E Larsson, K Rubin, L Klareskog. Homologous type II collagen induces chronic and progressive arthritis in mice. *Arthritis Rheum* 29:106, 1986.
- RA Ortmann and EM Shevach. Susceptibility to collagen-induced arthritis: cytokine-mediated regulation. *Clin Immunol* 98:109-118, 2001.
- WC Watson, AS Townes. Genetic susceptibility to murine collagen II autoimmune arthritis. Proposed relationship to the IgG2 autoantibody subclass response, complement C5, major histocompatibility complex (MHC) and non-MHC loci. *J Exp Med* 162:1878-1891, 1985.
- IK Campbell, JA Hamilton, IP Wicks. Collagen-induced arthritis in C57BL/6 (H-2b) mice: new insights into an important disease model of rheumatoid arthritis. *Eur J Immunol* 30:1568-1575, 2000.
- E Michaelsson, et al. T cell recognition of carbohydrates on type II collagen. *J Exp Med* 180:745-749, 1994.
- M Anderson, R Holmdahl. Analysis of type II collagen reactive T cells in the mouse. I. Different regulation of autoreactive vs. non-autoreactive anti-type II collagen T cells in the DBA/1 mouse. *Eur J Immunol* 20:1061-1066, 1990.
- R Reife, N Loutis, W Watson, K Hasty, J Stuart. SWR mice are resistant to collagen-induced arthritis but produce potentially arthritogenic antibodies. *Arthritis Rheum* 34:776-781, 1991.
- K Terato, et al. Collagen-induced arthritis in mice: synergistic effect of E.coli lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen. *Autoimmunity* 22:137-147, 1995.
- S Yoshino, E Sasatomi, Y Mori. Oral administration of lipopolysaccharide exacerbates collagen-induced arthritis in mice. *J Immunol* 163:3417-3422, 2000.
- BC Cole, MM. Griffiths. Triggering and exacerbation of autoimmune arthritis by the *Mycoplasma Arthritidis* superantigen MAM. *Arthritis Rheum* 36:994-1002, 1993.
- Y Takaoka, H Nagai, M Tanahashi, K Kawada. Cyclosporin A and FK-506 inhibit development of superantigen-potentiated collagen-induced arthritis. *Gen Pharmacol* 30:777-782, 1998.