Turkish Journal of Immunology - Open Access
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Turk J Immunol 2018; 6(1)30–36

A New Protocol for Collagen-Induced Local Arthritis Model in Balb/c Mice

Balb/c Farelerde Kollajen İle İndüklenmiş Lokal Artrit Modeli İçin Yeni Bir Protokol

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Abstract

Introduction: Numerous models have been described to study the pathogenesis of rheumatoid arthritis, and to develop new therapies; but each of these models has their own limitations. Nowadays, the collagen-induced arthritis (CIA) mouse model is the most commonly used model for rheumatoid arthritis studies in certain mouse strain like DBA/1 mouse. This study aimed to describe a new protocol for local induction of arthritis in Balb/c mice, including the monitoring of clinical arthritis and the protocols for histological examination of paws of mice.

Materials and Methods: For the induction of local arthritis in 40 Balb/c mice, they were immunized intra-articularly with type II collagen and subcutaneous complete Freund's adjuvant in 0 day. As the boost immunization, the animals had same injections on day 14. Mice were divided 4 groups, and they were clinically and histopathologically examined for arthritis on 0, 14, 21 and 30 days of arthritis induction.

Results: The first signs of local arthritis appeared in this model 1 week after boost immunization (day 21). The CIA induced paw clinically showed severe erythema and swelling all around the hindquarter on day 21 and 30. The paw reached almost 4 times thicker than the other paws and histopathological examination confirmed the clinical arthritis on day 21 and 30.

Conclusion: Using the protocol described, the investigators may reproducibly and economically induce a high incidence of local CIA in Balb/c mice. The described local CIA model may be used to unravel pathophysiological or immunological mechanisms of arthritis, and can also be used to study the effect of new therapeutics.

Keywords: Experimental model, rheumatoid arthritis, collagen-induced arthritis, Balb/c mouse

Öz

Giriş: Şimdiye kadar romatoid artritin patogenezini çalışmak ve yeni tedavi yöntemleri geliştirebilmek için çok sayıda model tanımlanmasına rağmen, bu modellerin sınırlılıkları vardır. Son günlerde romatoid artrit çalışmaları için en sık kullanılan model DBA/1 gibi belirli farelerde kollajen ile indüklenmiş artrit modelidir. Bu çalışmada Balb/c farelerde lokal artrit oluşturabilmek için yeni bir protokol tanımlanması amaçlanmıştır. Bu yeni protokol, çalışma esnasında farelerde oluşturulan klinik artrit semptomlarının gözlenmesini ve yine artritli fare pençesindeki histolojik inceleme prosedürünü içermektedir.

Gereç ve Yöntemler: Kırk adet Balb/c farede lokal artrit oluşturmak için, 0'ıncı günde fareler intraartiküler yolla tip II kollajen ve subkutan yolla complete Freund's adjuvant ile immünize edildi. İmmünizasyonun arttırılması için hayvanlara ilk uygulamanın 14. gününde aynı enjeksiyonlar uygulandı. Fareler dört gruba ayrıldı ve artrit indüklemesinin 0., 14., 21., ve 30. günlerinde klinik ve histopatolojik olarak incelendi.

Bulgular: Artritin ilk semptomları, ikinci uygulamadan bir hafta sonra gözlendi (21. gün). İndüklemenin 21. ve 30. günlerinde, kollajen ile indüklenmiş olan pençenin bulunduğu tüm arka ayak çevresinde, klinik olarak ciddi eritem ve şişkinlik gözlendi. Yine indüklemenin 21. ve 30. günlerinde, diğer pençelerle karşılaştırıldığında kollajen ile indüklenmiş olan pençe hemen hemen dört kat daha büyük kalınlığa ulaştı ve histolojik inceleme klinik artrit bulgularını doğruladı.

Sonuç: Araştırıcılar, tanımlanan metodu kullanarak, tekrarlanabilir ve ekonomik olarak Balb/c farelerde yüksek insidanslı lokal kollajen ile indüklenmiş artriti indükleyebilir. Tanımlanan lokal kollajen ile indüklenmiş artrit modeli, artritin fizyopatolojik veya immünolojik mekanizmasının çözümlenmesinde ve yeni tedavi etkilerinin çalışılmasında kullanılabilir.

Anahtar Kelimeler: Deneysel model, romatoid artrit, kollajen ile indüklenmiş artrit, Balb/c fare

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Received: Dec 21, 2017
Accepted: Mar 29, 2018

https://doi.org/10.25002/tji.2018.771

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects primarily the joints and is manifested as pain, stiffness, and synovitis (inflammation of the synovial membrane) leading in turn to articular destruction. Animal models of autoimmune arthritis have been considered valuable research tools for studying pathogenic mechanisms of this disease as well as testing new treatments. Several mouse models of arthritis have been developed to facilitate understanding of the pathogenesis of RA and development of new therapies. However, the collagen-induced arthritis model (CIA) has been the most widely studied model of RA. CIA was first described in rats[1] and subsequently shown to be inducible in susceptible strains of mice[2], following inoculation with type II heterologous collagen in complete Freund's adjuvant. Susceptibility to both RA and CIA is associated with the expression of specific class II major histocompatibility complex (MHC) molecules.[3,4] Earlier studies have shown that DBA/1 mice with the H-2q haplotype had the highest susceptibility, whereas the H-2b and H-2 d haplotypes like C57BL/6 and Balb/c mice, respectively, showed the lowest responsiveness to immunization with collagen and adjuvant. Class II MHC haplotypes and antigen presentation of specific collagen peptides to T cells also modulate the responsiveness of mice, but the adapted induction schedule and the use of a higher concentration of *Mycobacterium tuberculosis* have indicated that CIA can be induced in mice strains that were previously considered to be unresponsive.[5,6]

The establishment of a CIA model in Balb/c mice enables researchers to investigate the role of specific genes, receptors, or immune mediators such as cytokines in knockout or transgenic mice, which are often on a Balb/c background. In this way, avenues arise to study new targets for drug development. Therefore, the primary aim of the current study was to introduce new local CIA model in non-susceptible Balb/c mice for understanding pathophysiology of RA, and developing new therapies on the disease.

Material and Methods

Animals

Forty male Balb/c mice (H-2^d) (Experimental Animals Breeding and Research Center, Uludağ University, Bursa, Turkey), 6–8 weeks old were used. Before the experiments, they were housed five per cage at temperatures of (20–22°C) and humidity of (60–70%) in a controlled room set to a 12-h light: 12-h dark cycle and had access to standard mice chow and water *ad libitum*. The Animal Care and Usage Committee of Uludağ University, in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, approved all experimental procedures.[7]

Preparation of Collagen Type II Emulsion

Collagen is a fibrous protein that, at normal physiological conditions, is insoluble. Consequently, collagen type II from the bovine nasal septum (Sigma-Aldrich Co. LLC., cat. No. C7806-10 MG) was solubilized in a dilute solution of 0.1 M acetic acid (Sigma-Aldrich Co. LLC., cat. No. 38051-1EA) at the final concentration of 2 mg/ml collagen.

Induction Local CIA in Balb/c Mice

Arthritis was induced right paw of the mice. Left paw of the mice was used as a control. The mice were anesthetized with sevoflurane (2–4%/O_2 100%). The 10 µl Hamilton syringe was filled with the final concentration of 2 mg/ml collagen from the bovine nasal septum. Under the anesthesia, the injection area at the right knee joint and back of mice were cleaned with a wipe soaked in ethanol. A total of 10 µl collagen from bovine nasal septum emulsion (2 mg/ml) was injected carefully into the right knee joint with glass Hamilton syringe and 25G needles for intraarticular (i.a.) injection. Just after this, a total of 100 µl Complete Freund's adjuvant (CFA, *Mycobacterium tuberculosis*, heat killed and dried; Sigma-Aldrich Co. LLC., cat. No. F5881-6X10ML) emulsion is injected at the back of mice using the syringe with 25G needles subcutaneously (s.c.). When injected properly, a white lobule should be visible at the site of injection. The mice put back in their cages and expected to recover from anesthesia after the injections were completed. At day 14 after the first injection, the mice were injected i. a. with 10 µl of collagen from the bovine nasal septum (2 mg/kg) and s.c. with 100 µl CFA emulsion for boost injection.

Monitoring of Local Arthritis

Local arthritis was monitored using a clinical arthritis scoring system and paw swelling was measured with help of a caliper. Measurements were taken on day 0, 14, 21 and 30 days of induction as arthritis induced paws (right paw) and non-arthritis induced paws (left paw). Scoring

was initiated on 0 day after administration of primary dose of collagen from the bovine nasal septum and CFA Table 1 shows how scores are assigned.

Table 1. Hindquarter swelling scores in Balb/c mice

Scores	Description
0	Normal
1	Mild erythema around joint
2	Moderate erythema and mild swelling around joint
3	Severe erythema and moderate swelling all around the hindquarter
4	Severe erythema and swelling all around the hindquarter

To measure paw thickness of collagen treated hind ankle joint, mice were restrained by holding by the scruff of the neck and tail and then paw thickness were measured by using caliper. Always hold the caliper in the same way during each measurement.

Decalcification and Histological Examination of Mice Paws

At the end of the study, the mice were anesthetized with sevoflurane (2–4%/O_2 100%). They were sacrificed by cervical dislocation under the anesthesia. The hind paws of the mice were cut of the level of hips to keep the knee joint intact for histopathological examination. The skin of the hind paws was removed. After that, the tissue samples were fixed in 10% formalin solution for 48 hours at 37°C in an incubator.

The samples were put in decalcifying solution (10%, Ethylenediaminetetraacetic acid) for two weeks following the fixation process was completed. The decalcifying solution was changed every other day. Decalcified tissue samples were then processed routinely for histological examination. Briefly, 5 µm tissue sections were cut and mounted glass slides. All tissue sections were stained with Haematoxylin-Eosin (H&E) and evaluated light microscopically.

Results

Ten animals were used for day 0 observation. Those animals had first immunization but they did not show any clinical or histopathological sign on day 0 (Fig. 1, A, B, C). Second ten mice were sacrificed just after boost immunization (day 14). Those paws of mice showed clinically mild erythema around joint and histopathologically mild inflammatory cell infiltration (Fig. 1, D, E, F).

One week after the boost immunization (day 21), the paw clinically started to show severe erythema and swelling all around the right hindquarter of Balb/c mice on both days 21 (Fig. 1G, n=10) and these signs continued through day 30 (Fig. 1J, n=10) when they were compared the control paws. The local CIA arthritis induced paws (right) reached 4 times thicker than the non-arthritis induced paws (left) at the first week of the boost immunization (Fig. 2). According to table 1 scoring the local CIA arthritis induced paw (right) and the control paw (left) of the mice were scored as “4” and “0”, respectively, on both days 21 (Fig. 1, G, H) and 30 (Fig. 1, J, K). Table 2 demonstrated the time scales of clinical scores of the CIA induced paws and control paws. Moreover, the local CIA arthritis induced paws demonstrated histopathologically inflammatory cell infiltrations, palisading of synovial cells and proliferation of synovial cells in synovial membranes on both days 21 (Fig. 1, I) and 30 (Fig. 1, L). Neovascularization, existence of synovial granulation tissue (pannus) extended into the cartilage surface, chondrocytes necrosis in cartilage, bone lysis and fibrinous material within joint space were also noticed on both days 21 (Fig. 1, I) and 30 (Fig. 1, L).

Table 2. Time scales of clinical scores of the CIA induced paws and control paws

Days	CIA induced Paw (Right)	Control Paw (Left)
0	0	0
14	1	0
21	4	0
30	4	0

Discussion

In this study, we showed that Balb/c mice were immunized with i.a. collagen from the bovine nasal septum and with s.c. CFA containing the amount of *Mycobacterium tuberculosis*. For boost immunization, the same mice had the same dose and route injections at day 14 after the first injection. After the boost immunization, the paw showed clinically and histologically long-lasting severe arthritis signs.

Collagen Type II-dependent both a T-cell and B-cell-specific response involves to the immunopathogenesis of CIA. The immunodominant T-cell determinants of Collagen Type II that mediate CIA have been identified

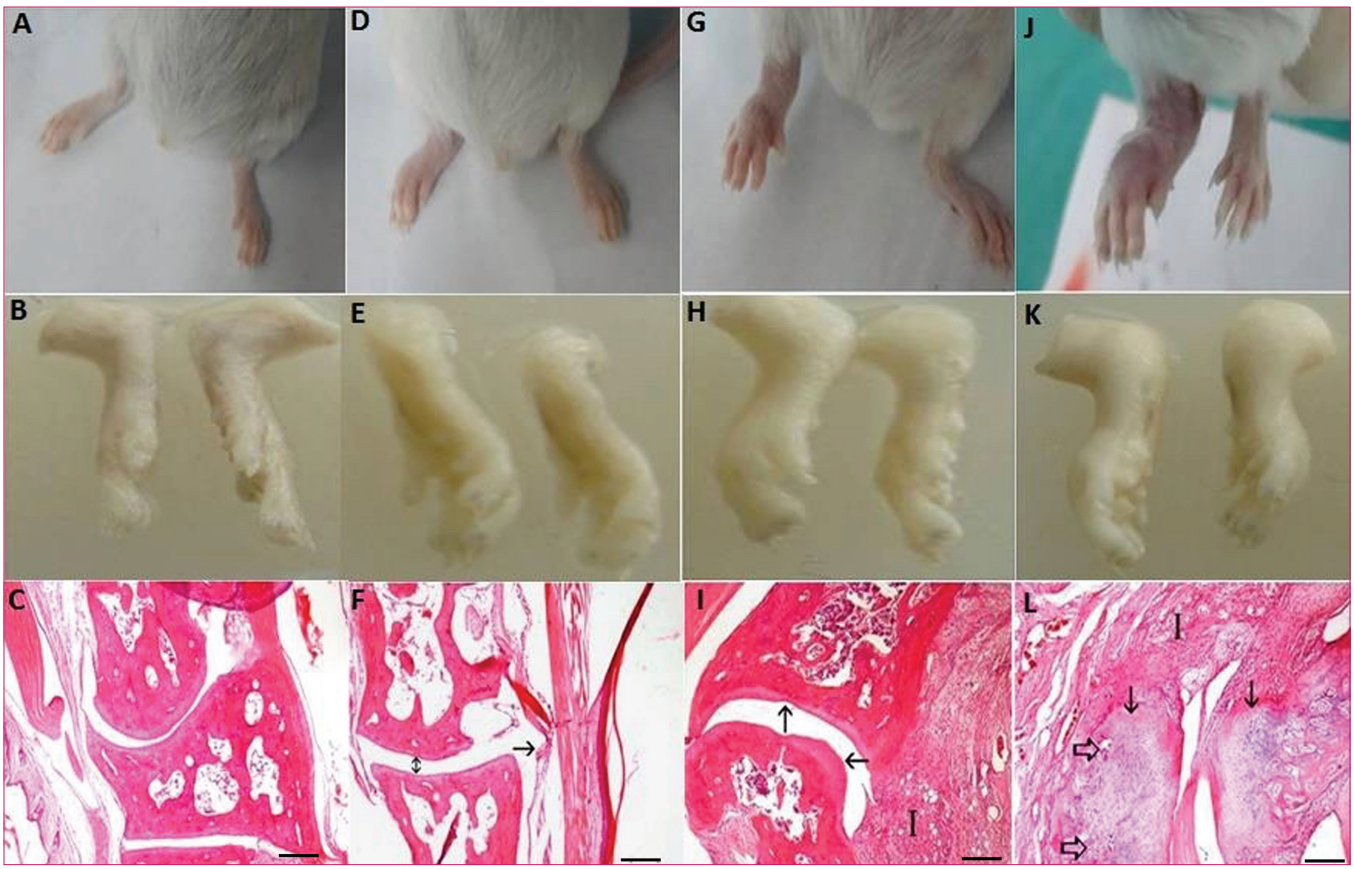

Figure 1. Time dependent arthritis severity CIA induced arthritis in Balb/c mice. Day 0: A, macroscopic joint view; B, decalcified joints; C, microscopic view of articular cartilage 4x obj., hematoxylin-eosin staining. Day 14: D, macroscopic joint view; E, decalcified joints; F, microscopic view of articular cartilage 4x obj., hematoxylin-eosin staining. Day 21: G, macroscopic joint view; H, decalcified joints; I, microscopic view of articular cartilage 4x obj., hematoxylin-eosin staining. Day 30: J, macroscopic joint view; K, decalcified joints; L, microscopic view of articular cartilage 4x obj., hematoxylin-eosin staining. In hematoxylin-eosin staining: inflammatory cell infiltrations, palisading of synovial cells and proliferation of synovial cells in synovial membranes *(horizontal arrows)*; neovascularization and existence of synovial granulation tissue *(hollow arrows)*; chondrocytes necrosis and bone lysis *(vertical arrows)*. The bar shows 250 µm.

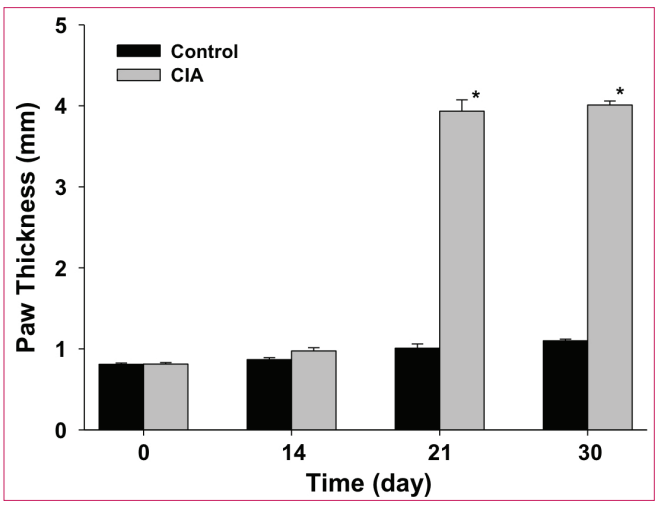

Figure 2. The paw thickness local CIA induced arthritis in Balb/c mice. To monitor the severity of the arthritis, paw swelling was measured by using a caliper. Measurements were taken on day 0, 14, 21 and 30 days after first immunization. Data is given as means ± S. E. M. of ten measurements. Statistical analysis was performed by using Student T test. *Shows significantly different from that of the control. p values were 0.0000123 and 0,000010 for day 21 and 30, respectively. Control is the non arthritis-induced paw *(left paw)* thickness and CIA is arthritis-induced paw *(right paw)* thickness.

for most of the class II molecules that are associated with susceptibility to this experimental disease[8–12] and a few have been studied in detail for their interaction with the class II molecule and T-cell receptor.[13,14] Similarly, B-cell determinants targeted by the antibody response to Collagen Type II have also been identified[15,16], and

there is some evidence that antibodies from RA patients target the same areas of the Collagen Type II molecule as those from CIA.[17] Identification of pathogenic B-cell determinants has proven to be more difficult owing to the requirement that the pathogenic antibodies must be able to bind to the triple helical native Collagen Type II. The pathogenesis of CIA is mediated, in a large part, by CII-specific antibody that binds to the cartilage, and is capable of fixing complement. In the present study Balb/c mice also required immunization with CFA for elicitation of a high incidence of arthritis. This is likely due to the stimulatory capacity of heat-killed Mycobacterium in the adjuvant, and its promotion of the inflammatory response that initiates an immune response to Collagen Type II. This concept is supported by the fact that a higher than usual concentration of the heat-killed *Mycobacterium tuberculosis* is required to elicit a high incidence of disease in the CIA model.

Animal models of autoimmune arthritis have proven to be valuable research tools for the study of pathogenic mechanisms of RA as well as for testing new therapies. Several mouse models of arthritis have been established.[18] While each of these described models has advantages and disadvantages, CIA has been the most widely studied model of RA. It shares several pathological features with RA, and Collagen Type II is a major protein in cartilage, the target tissue of RA. Additionally, of the antigen-defined models that are based on cartilage proteins, it has the shortest duration between immunization and disease manifestation. The CIA model has been used extensively to identify potential pathogenic mechanisms of autoimmunity, including the role of individual cell types in disease onset and progression, as well as to design and test new therapeutics. In recent years, the CIA model has been instrumental in the testing and development of the new biologically based therapeutics, such as those that target tumor necrosis factor-α, a cytokine produced by macrophages and T cells that is a dominant inflammatory mediator in the pathogenesis of RA. The development of these biologically based therapies has revolutionized the treatment of RA.

Susceptibility to CIA is linked to major histocompatibility complex-class II molecules which respond to individual species of type II collagen used for immunization.[19] It is known that the major histocompatibility complex and non-major histocompatibility complex genetic backgrounds make the mice strain highly susceptible to collagen antibody induced arthritis. Due to the specific genetic background, the Balb/c strain shows a strong predisposition toward collagen antibody induced arthritis or proteoglycan-induced arthritis but not CIA.[20] On the other hand, Balb/c mice are capable of producing arthritogenic antibodies, suggesting that CIA is not only restricted by MHC types.[21] In addition, despite the efforts of companies to maintain genetically homogenous inbred mouse colonies, there are differences among BALB/c colonies or substrains (for example, in body weight, size of littermates, and the composition of microbiota) maintained at different locations by the same vendor. According to the online public database of The Jackson Laboratory (Bar Harbor, ME, USA)[22], there is at least 492 single nucleotide polymorphism (SNP) differences between their two inbred BALB/cJ and BALB/cByJ colonies. Some of these polymorphisms or mutations may influence the predisposition of the mice to CIA.[20]

Strain-specific differences in cytokine production provide us opportunities to understand the mechanisms responsible for cytokine control of susceptibility and resistance to autoimmune disease. It was reported that the bias toward a Th2 response in BALB/c mice might be regulated at two stages of the immune response.[23–25] Initially, there is an increase in the frequency of Th2 cells producing IL-4, which is conducted by a locus on chromosome 16. Then, loss of IL-12 responsiveness occurs due to the decrease in IL-12R expression, which is influenced by a locus on chromosome 11. Resistance to autoimmune disease in this model found to be correlated with differentiation of naive T cells into Th2-type cells.[25] Despite the predisposition of BALB/c mice to a Th2-type response, Finnegon et al.[26] demonstrated that induction of arthritis in Balb/c mice by proteoglycan is a Th1-type disease. The effectiveness of IL-4 treatment was particularly striking in their study since in other models of arthritis, treatment in a similar manner with IL-4 was not able to ameliorate arthritis. That study suggested that levels of endogenous IL-4 in BALB/c mice might increase their responsiveness to Th2 cytokine therapy. Similar studies should be employed in distinct BALB/c colonies or substrains to find out colony- or substrain-specific responses in autoimmune disease models.

Although it is accepted that Balb/c mice belong to strains of mice regarded to have low susceptibility to CIA, our results demonstrated that if mice are immunized with i.a. collagen from bovine nasal septum and with s.c. CFA at

day 0, and are boosted with same regime on day 14, Balb/c mice developed arthritis with high incidence in a week.

Conclusion

Collectively, these data have enabled researchers to study a wide range of pathogenic mechanisms in this described local CIA model, as well as to design and test novel therapeutics.

Acknowledgements

This study was supported by The Scientific and Technical Research Council of Turkey (TUBITAK-COST Grant No 113S375) under Cost Action BM1305. COST is supported by the EU Framework Programme Horizon 2020.

Ethics Committee Approval: The Animal Care and Use Committee of Uludağ University, in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, approved all experimental procedures.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - MY, HBO; Supervision MY; Resources - HBO; Data Collection and/or Processing - GG, MK, AA, KÇ; Analysis and/or Interpretation - GG, AA, MY; Literature Search - GG, MY, KÇ; Writing Manuscript- GG, MY, HBO; Critical Review - MY, HBO.

Conflict of Interest: No conflict of interest was declared by the authors.

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