

T-Cell Proliferation Grade Type I and Type II Collagen

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INTRODUCTION

T-cells play a crucial role in the induction of collagen-induced arthritis (CIA), since type II collagen is a T-cell dependent antigen. However, it is difficult to study T-cell responses to type II collagen even in CIA high responder animals by T-cell proliferation assay using spleen and lymph node cells due to several reasons: 1) Collagen is not a strong T-cell antigen and T-cell population specific to collagen is low. 2) T-cell proliferation responses will be masked due to the high background levels caused by polyclonally stimulated B-cells with adjuvant. B-cells, even from animals immunized with incomplete adjuvant, proliferate spontaneously. 3) Macrophages activated by adjuvant components might release a large amount of immuno-suppressive cytokines, which inhibit T-cell proliferation. 4) Pepsin, which is commonly used for solubilizing collagen and common contaminant in collagen preparations, is a strong T-cell antigen, and creates significantly high false positive results. In order to determine true T-cell responses to collagen, it is important to use a T-cell enriched fraction from spleen or lymph node cells, and to use highly purified, pepsin free collagen preparations as a T-cell stimulation antigen in vivo.

Type II collagen is particularly used as an immunizing antigen for inducing arthritis in rodents and monkeys. However, depending upon the research purposes, animals may be immunized with type I collagen or receive collagen implants. Chondrex provides various species of highly purified T-cell grade type I collagen as well as type II collagen for various purposes of immunological studies on collagen and collagen related materials (i.e. T-cell proliferation assays and establishing T-cell lines and T-cell hybridoma cells). T-cell grade type I and type II collagen (0.5 mg/vial, lyophilized) is supplied in a heat-denatured form. In addition, for T-cell epitope studies CNBr-digested type II collagen fragments can be provided upon request. Type I collagen can be used as a control antigen for T-cell epitope studies in the CIA model and other autoimmune models, since type I and type II collagen share more than 80% amino acid sequences, but their immunological features differ significantly.

PROCEDURE FOR IN VITRO T-CELL PROLIFERATION ASSAY

Immunization

Immunize animals such as mice, rats or monkeys with type II collagen emulsified with CFA (catalog # 7001) or IFA (catalog # 7002). Follow the immunization protocol for inducing collagen-induced arthritis in individual animals. Collect spleen or lymph node cells on day 8-12 after immunization. In the case of monkeys, use peripheral blood mononuclear cells or an underarm lymph node removed surgically.

Note 1: It is not necessary to use the immunization protocol for inducing CIA in these animals, but it might be convenient to follow the protocol. For example, immunize mice with 100 µg of type II collagen emulsified with CFA, rats with 200 µg of type II collagen emulsified in IFA and monkeys with 250 µg of type II collagen emulsified with CFA.

Note 2: T-cell proliferation response and their epitope specificity are not dependent on the conformational structure of type II collagen or the type of adjuvant. Identical T-cell responses are observed in mice immunized with either native or denatured collagen emulsified with both CFA and IFA. Therefore, for T-cell studies, denatured collagen and even CB peptide fragments can be used as immunizing antigens. On the other hand, antibody specificity and induction of arthritis are strongly dependent on the conformational structure of type II collagen and the type of adjuvant (see "Successful Induction of Arthritis in Mice" and "Successful Induction of Arthritis in Rats").

Purification of T-Cells

1. Pack 0.6 g of pre-washed nylon-wool into a 10 mL syringe, soak with PBS and autoclave for 20 minutes. Replace the PBS with Dulbecco's Minimum Essential Medium (DMEM) supplemented with 1% homologous normal serum, penicillin-streptomycin (1X), and 2-mercaptoethanol (2-ME - 5×10^{-5} M). This is called complete DMEM.

Note: Do not use fetal calf or bovine serum in any case, since T-cells spontaneously react to heterologous serum components. In the case of monkeys, use Protein A-adsorbed autologous serum or plasma (Protein A removes immunoglobulins including antibodies against collagen).

2. Prepare a single cell suspension from spleen or lymph node cells from mice immunized with type II collagen (or type I collagen), wash 3 times with DMEM and then suspend the cells in 1-2 mL of complete DMEM (approximately 1×10^8 cells/mL).
3. Load the cell suspension onto the column. Incubate the column for 30 minutes in a CO₂ incubator at 37°C. Elute unbound cells with 20 mL DMEM and then centrifuge at 800 rpm for 8 minutes. Suspend the enriched T-cells at $4-8 \times 10^6$ cells/mL in complete DMEM.

Preparation of Antigen Presentation Cells (APC)

1. Use spleen or thymus cells obtained from young, normal congenic animals (6-8 weeks old) as APC. Wash the cells with DMEM 3 times and suspend at 1×10^7 cells/mL in complete DMEM.

Note: In the case of monkeys, use Mytomycin C (an inhibitor of DNA synthesis) treated peripheral blood mononuclear cells (PBMC) isolated by the Ficoll-Hypaque method as APC.

2. Irradiate the cells with 2000 rad = 20 Gy.

Note: Do not disturb the cells after irradiation and treatment with Mytomycin C (50 µg/mL for 1 hour).

T-Cell Proliferation Assay

1. Dissolve T-cell grade type II collagen (or type I collagen) at 1 mg/mL with warm PBS or DMEM and then centrifuge at 10,000 rpm for 3 minutes at room temperature. Transfer the supernatant to a sterile tube and store at -20°C until needed.

Note: T-cell grade type II collagen (or type I collagen) has been denatured by heating. Type II collagen (or type I collagen) used for T-cell stimulation should be denatured, since APC are not capable of processing native type II collagen (or type I collagen) in the short culture period.

2. Add 0.1 mL of enriched T-cells ($4-8 \times 10^6$ cells/mL), 0.1 mL of APC (1×10^7 cells/mL) and 10-20 µL of 1 mg/mL T-cell grade type II collagen (or type I collagen) in a 96-well flat bottom plate. Culture the cells in a CO₂ incubator at 37°C with 5% CO₂ for 3 days.

Note 1: In the case of monkeys, optimization of T-cells/APC ratio (in general, an equal number of T-cell and APC recommended) is critical to obtain the maximum T-cell responses to collagen.

Note 2: Do not use round bottom plates.

3. Add ³H-labeled thymidine (0.5-1.0 mCi/well) on day 3 and culture for an additional 15-18 hours. Harvest the cells on a glass filter and assay for the incorporated ³H by scintillation counting.