J. Pharm. Pharmacol. 1996, 48: 702–705
Received March 1, 1996
Accepted April 11, 1996

Suppression of Adjuvant Arthritis in Rats by Oral Administration of Type II Collagen in Combination with Type I Interferon

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Abstract

The oral administration of 3 μg but not 300 μg of type II collagen (CII) significantly suppressed adjuvant arthritis in rats that was induced by immunization with *Mycobacterium tuberculosis*. Feeding 5000 units of type I interferon was also effective in downregulating the disease. More suppression of adjuvant arthritis was observed when both CII and interferon were orally given. Delayed-type hypersensitivity responses to *M. tuberculosis* were inhibited in interferon- but not in CII-fed animals. There were no delayed responses to CII in *M. tuberculosis*-immunized rats fed either CII or interferon. However, the delayed response to *M. tuberculosis* plus CII was significantly reduced by CII as well as interferon given orally. Feeding both CII and interferon was more effective in suppressing the delayed responses to *M. tuberculosis* plus CII. A similar suppression was observed in proliferative responses of lymph-node cells to *M. tuberculosis*, CII, or *M. tuberculosis* plus CII in-vitro.

These results suggest that the suppression of adjuvant arthritis by oral administration of CII is due to tolerance to CII in which a bystander suppression mechanism appears to be involved. Orally administered interferon seems to suppress nonspecifically cellular immune responses. The oral administration of CII in combination with interferon may be a novel way to treat T cell-mediated diseases.

Adjuvant arthritis in rats, which resembles human rheumatoid arthritis in certain respects, can be induced by immunization with *Mycobacterium tuberculosis* (Pearson 1956; Pearson & Wood 1963). Adjuvant arthritis also can be passively transferred to naive normal rats with lymph node or spleen cells from animals with adjuvant arthritis (Pearson & Wood 1964), or T cell clones specific for *M. tuberculosis* (van Eden et al 1985), suggesting that autoimmune responses to joint components including cartilage proteoglycan that cross-react with *M. tuberculosis* are involved in the pathogenesis of the disease (van Vollenhoven et al 1988).

Oral administration of antigen induces a state of immunological unresponsiveness, termed oral tolerance. Recent studies showed that orally fed type II collagen (CII) suppressed collagen-induced arthritis in rats (Thompson & Staines 1985) and mice (Nagler-Anderson et al 1986) that was induced by immunization with CII. In contrast, adjuvant arthritis and delayed-type hypersensitivity responses to the disease-causing antigen *M. tuberculosis* are not affected by feeding *M. tuberculosis*, indicating that no oral tolerance to *M. tuberculosis* is induced (Higgins & Weiner 1988). However, adjuvant arthritis is significantly downregulated by oral CII, although a mechanism of the disease suppression remains to be elucidated.

Type I interferon downregulates proliferative responses in-vitro (Derbyshire 1991) and oral administration of interferon has been shown to suppress experimental autoimmune encephalomyelitis in animals that is induced by immunization with myelin basic protein (Burns et al 1993). Thus, orally fed interferon also may modulate adjuvant arthritis.

In the present study we show that interferon as well as CII administered orally suppressed the development of adjuvant arthritis. In addition, feeding CII in combination with interferon was more effective in suppressing the disease than giving CII or interferon alone. The delayed-type hypersensitivity and proliferative responses to *M. tuberculosis* plus CII, but not to *M. tuberculosis* alone were inhibited in CII-fed rats, suggesting that bystander suppression may be the mechanism by which adjuvant arthritis is downregulated by the fed antigen.

Materials and Methods

Animals

Inbred female Lewis rats, 120–130 g, were obtained from Charles River Japan, Inc. (Shiga, Japan). They were housed two per cage and had free access to a standard rodent chow and water.

Induction of adjuvant arthritis

Adjuvant arthritis was induced by an intradermal injection into the tail base of 0·1 mL complete Freund's adjuvant containing 10 mg mL^{-1} *M. tuberculosis* (H73Ra; Difco Laboratories, Detroit, MI, USA). To evaluate the severity of arthritis, the lesions of the four paws were each graded from 0 to 4 according to the increasing extent of erythema and oedema of the periarticular tissue as described by Wood et al (1969). The maximum possible score is 16.

Administration of CII and interferon

Three or three hundred micrograms CII extracted from native calf articular cartilage (Funakoshi Co., Tokyo, Japan), dissolved in 1 mL 0·01 M acetic acid, or 5000 units of rat type I (α/β) interferon (Lee Biomolecular Research Inc., San Diego, CA, USA), dissolved in 1 mL phosphate-buffered saline, was orally administered through a syringe fitted with an 18G ball-point needle on days −5, −4, −3, −2, and −1 before immunization with *M. tuberculosis*. One millilitre of 0·01 M acetic acid and 3 μg of hen egg lysozyme were given at the same time as controls.

Measurement of delayed-type hypersensitivity

Twenty micrograms of *M. tuberculosis*, CII, or both *M. tuberculosis* and CII dissolved in 50 μL 0·001 M acetic acid was

subcutaneously injected into the right ear of rats immunized with *M. tuberculosis* on day 10. As a vehicle control, 50 μL 0·001 M acetic acid alone was injected into the left ear. The thickness of the right and left ears were measured using dial gauge calipers calibrated with 0·01 mm graduations (Ozaki MFG Co., Tokyo, Japan) immediately before and 48 h after the challenge injection of *M. tuberculosis*, CII or both *M. tuberculosis* and CII. There was minimal ear swelling after 48 h in response to *M. tuberculosis* or CII in nonimmunized rats. The increase in left ear thickness was subtracted from the increase in right ear thickness, to determine swelling attributable to the specific response to antigen.

Proliferation assay

Ten days after immunization with *M. tuberculosis*, rats were killed, the inguinal lymph nodes were removed, and single cell suspensions were prepared. To each microwell was added 5×10^{-5} cells in 0·1 mL RPMI 1640 containing 2% glutamine, 1% penicillin/streptomycin, 5×10^{-5} M mercaptoethanol, and 1% autologous rat serum, followed by addition of 0·1 mL 5 μg mL^{-1} *M. tuberculosis*, 20 μg mL^{-1} CII, or both 5 μg mL^{-1} *M. tuberculosis*, 20 μg mL^{-1} CII. The cells were cultured for 72 h, each well was pulsed with 0·5 μCi tritiated thymidine, and cells were cultured for another 16 h. Cultures were harvested onto fibreglass filters using a multiharvester and counted using standard liquid scintillation techniques. All cultures were performed in quadruplicate in flat-bottomed 96-well plates and the stimulation index of proliferative responses of lymph node cells was calculated as counts min^{-1} of cells with antigen/counts min^{-1} of cells without antigen.

Results

Effect of oral administration of CII and interferon on adjuvant arthritis

To investigate the effects of oral administration of CII and interferon on adjuvant arthritis, the materials and controls (acetic acid and hen egg lysozyme) were orally given on days −5, −4, −3, −2, and −1 before immunization with *M. tuberculosis*. The results showed that rats fed controls had a sign of arthritis on day 12 and thereafter severe joint inflammation developed rapidly (Fig. 1). On the other hand, animals given orally 3 μg CII or interferon had a significantly reduced

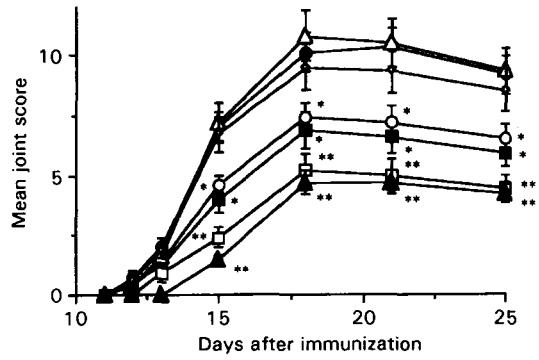

Fig. 1. Effect of oral administration of CII, interferon, or CII plus interferon on adjuvant arthritis in rats. Animals were fed 3 (■) or 300 (△) μg CII, 5000 units interferon (○), or both 3 μg CII and 5000 units interferon (□), or both 300 μg CII and 5000 units interferon (▲) on days −5, −4, −3, −2, and −1 before immunization with *M. tuberculosis*. Acetic acid (●) and 3 μg lysozyme (◇) were given as controls. Adjuvant arthritis was induced on day 0 as described in Materials and Methods. Bars show the mean ± s.e.m. of seven rats. $*P < 0.05$ and $**P < 0.01$ vs acetic acid-fed group, by Student's *t*-test.

severity of arthritis. When both 3 μg CII and interferon were orally administered, more marked suppression of arthritis was observed. In contrast, the larger dose, 300 μg, of CII given orally did not affect the development of arthritis, although the combination of 300 μg CII and interferon markedly suppressed joint oedema to the extent shown in rats fed 3 μg CII together with interferon.

Effect of oral administration of CII and interferon on delayed-type hypersensitivity responses to M. tuberculosis, CII, or both M. tuberculosis and CII

Since adjuvant arthritis appears to be mediated by T cells specific for *M. tuberculosis* (Pearson & Wood 1964; van Eden et al 1985), we tested whether feeding CII and interferon could downregulate the T cell-mediated hypersensitivity, delayed-type hypersensitivity, to *M. tuberculosis* in the ear. Table 1 shows the results. There was no difference in delayed-type hypersensitivity responses to *M. tuberculosis* between rats fed acetic acid, lysozyme and 3 μg CII. In contrast, when interferon was orally administered, the delayed-type hypersensitivity was significantly suppressed. A similar degree of suppression of the

Table 1. Effect of oral administration of interferon and CII on delayed-type hypersensitivity responses to *M. tuberculosis*, CII, or *M. tuberculosis* plus CII.

Feeding	Ear swelling ($\times 10^{-2}$ mm)		
	M. tuberculosis	CII	*M. tuberculosis* plus CII
Acetic acid	102 ± 8·9	4 ± 2·4	95 ± 7·5
Lysozyme	90 ± 10·9	3 ± 1·9	98 ± 9·0
CII	98 ± 10·5	5 ± 2·8	71 ± 6·0*
Interferon	70 ± 8·4*	3 ± 1·8	75 ± 5·3*
CII/interferon	66 ± 10·3*	5 ± 2·0	44 ± 6·6**

Rats were fed 3 μg CII, 5000 units interferon, or both 3 μg CII and 5000 units interferon on days −5, −4, −3, −2, and −1 before immunization with *M. tuberculosis*. Acetic acid and 3 μg lysozyme were given as controls. Ear delayed-type hypersensitivity responses to *M. tuberculosis*, CII, or *M. tuberculosis* plus CII were determined on day 10 as described in Materials and Methods. Values are expressed as the mean ± s.e.m. of seven rats. $*P < 0.05$ and $**P < 0.01$ vs acetic acid-fed group, by Student's *t*-test.

Table 2. Effect of oral administration of interferon and CII on the proliferative response to *M. tuberculosis*, CII, or *M. tuberculosis* plus CII,

Feeding	Stimulation index		
	M. tuberculosis	CII	*M. tuberculosis* plus CII
Acetic acid	18·6 ± 2·44	1·2 ± 0·08	20·4 ± 1·82
Lysozyme	20·1 ± 1·98	1·0 ± 0·05	18·8 ± 2·17
CII	19·5 ± 2·24	1·3 ± 0·06	11·5 ± 1·04
Interferon	9·4 ± 1·03	1·1 ± 0·08	10·6 ± 1·21
CII/interferon	11·2 ± 0·81	1·3 ± 0·06	5·8 ± 0·62

Rats were fed 3 μg CII, 5000 units of interferon, or both 3 μg CII and 5000 units interferon on days −5, −4, −3, −2, and −1 before immunization with *M. tuberculosis*. Acetic acid and 3 μg lysozyme were given as controls. Proliferative responses of inguinal lymph node cells to *M. tuberculosis*, CII, or *M. tuberculosis* plus CII were determined on day 10 as described in Materials and Methods.

response was observed in animals fed both interferon and CII. There were no significant delayed hypersensitivity responses to CII in all groups tested. On the other hand, when delayed-type hypersensitivity to both *M. tuberculosis* and CII was examined, rats administered orally either CII or interferon had significantly reduced ear swelling. The oral administration of CII in combination with interferon appeared to give more suppressive effects on the responses to *M. tuberculosis* plus CII than that of CII or interferon alone.

Effect of oral administration of CII and interferon on proliferative responses to M. tuberculosis, CII, or both M. tuberculosis and CII

We also studied the effect of orally fed CII and interferon on proliferative responses to *M. tuberculosis* in-vitro (Table 2). The oral administration of either lysozyme or CII did not affect the proliferation of lymph node cells to *M. tuberculosis*. In contrast, marked suppression of the proliferative response was observed in rats fed interferon and also in those given orally CII plus interferon. There was no significant proliferation to CII in all groups examined. On the other hand, the stimulation with both *M. tuberculosis* and CII was effective in suppressing significantly the growth of lymph node cells from animals given orally either CII or interferon, but not lysozyme. More marked suppression of the proliferation was observed in both CII and interferon fed rats.

Discussion

The present study demonstrates that oral administration of 3 but not 300 μg CII is effective in suppressing the T cell-mediated disease adjuvant arthritis. This supports the previous finding that adjuvant arthritis was downregulated by orally fed CII at the doses of 3 and 30 μg, but not at 300 and 1000 μg (Higgins & Weiner 1988). However, those workers did not show mechanisms of the suppression, although it was suggested that oral tolerance to CII might be induced following its administration, since orally given antigens had been shown to be tolerogenic (Well 1911) and imununity to CII to be induced in rat with adjuvant arthritis (Trentham et al 1980). Our results indicate that oral tolerance to CII is induced following oral administration of the antigen. In addition, the present report indicates that a bystander suppression mechanism appears to be involved in oral tolerance, since rats fed CII had significantly reduced ear delayed-type hypersensitivity and proliferative responses to *M. tuberculosis* plus CII, but not to *M. tuberculosis* alone. Furthermore, in the present study there were no delayed-type hypersensitivity and proliferative responses to CII in *M. tuberculosis*-immunized rats, suggesting that CII might not be critically involved in the pathogenesis of adjuvant arthritis. We do not know why the higher dose (300 μg) of orally administered CII failed to affect adjuvant arthritis. Collagen-induced arthritis is also suppressed by feeding smaller doses of CII, but not by larger doses (Thompson & Staines 1985; Nagler–Anderson et al 1986). Thus, oral tolerance appears to be induced by small doses of oral antigens.

Interferon injected systemically has an inhibitory effect on proliferative responses (Derbyshire 1991), and recent investigation showed that oral administration of 5000 units interferon markedly suppressed experimental autoimmune encephalomyelitis in mice that was induced by immunization with myelin basic protein (Burns et al 1993). The present study also showed that the dose of the cytokine was effective in suppressing adjuvant arthritis, confirming that interferon is active by the oral route. The suppression of the disease by the oral cytokine appears to be due to the reduction of cellular immune responses to *M. tuberculosis*, since orally administered interferon suppressed the *M. tuberculosis*-induced delayed-type hypersensitivity response in the ear and proliferation to the antigen in-vitro.

Our results also demonstrate that the oral administration of CII in combination with interferon is more effective in suppressing adjuvant arthritis than that of CII or interferon alone. Especially, it is of note that 300 μg CII given orally together with interferon suppressed adjuvant arthritis markedly despite no influence on the disease of the oral administration of that dose of CII alone.

The suppression of adjuvant arthritis by orally fed CII, interferon, or CII plus interferon suggests that ingested proteins may be absorbed from the gut. It has been shown that membranous epithelial cells in Peyer's patches function for protein uptake (Wolf & Bye 1984) and dietary antigens, either undegraded or partially degraded, are absorbed from the small intestine (Husby et al 1986; Bruce & Ferguson 1987).

Recent clinical trials demonstrated that the oral administration of CII to patients with rheumatoid arthritis was effective in decreasing joint swelling and disease index (Trentham et al 1993). There is significantly reduced interferon production

observed in patients with rheumatoid arthritis (Seitz et al 1987). Therefore, oral administration of CII in combination with interferon may be a novel means to treat the disease more effectively.

References

Bruce, M. G., Ferguson, A. (1987) Oral tolerance produced by gut processed antigen. Adv. Exp. Med. Biol. 216A: 721–731

Burns, D. L., Scott, M., Phillips, J. T., Brod, S. A. (1993) Oral administration of type I interferons prevents sensitization to MBP in acute EAE. J. Immunol. 150: 195A

Derbyshire, J. B. (1991) Suppression of lymphocyte blastgenesis by porcine interferon-alpha. Res. Vet. Sci. 50: 376–378

Higgins, P. L., Weiner, H. L. (1988) Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. J. Immunol. 140: 440–445

Husby, S., Jensenius, J. C., Svehag, S.-E. (1986) The influence of intestinal processing on the immunogenicity and molecular size of absorbed, circulating ovalbumin in mice. Immunology 59: 295–300

Nagler-Anderson, C., Bober, L. A., Robinson, M. E., Siskind, G. W., Thorbecke, G. J. (1986) Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen. Proc. Natl. Acad. Sci. USA 83: 7443–7446

Pearson, C. M. (1956) Development of arthritis, periarthritis and periotitis in rats given adjuvant. Proc. Soc. Exp. Biol. Med. 91: 95–101

Pearson, C. M., Wood, F. D. (1963) Studies of arthritis and other lesions induced in rats by the injection of Mycobacterial adjuvant. VII. Pathologic details of the arthritis and spondylitis. Am. J. Pathol. 42: 73–95

Pearson, C. M., Wood, F. D. (1964) Passive transfer of adjuvant arthritis by lymph node or spleen cells. Am. J. Pathol. 120: 547–560

Seitz, M., Napierski, R., Augustin, I. R., Hunstein, W., Kirchner, H. (1987) Reduced production of interferon alpha and interferon gamma in leucokyte cultures from patients with active rheumatoid arthritis. Scand. J. Rheumatol. 16: 257–262

Thompson, H. S. G., Staines, N. A. (1985) Gastric administration of type II collagen delays the onset and severity of collagen-induced arthritis in rats. J. Clin. Invest. 62: 581–586

Trentham, D. E., McCure, W. J., Susman, P., David, J. R. (1980) Autoimmunity to collagen in adjuvant arthritis of rats. J. Clin. Invest. 66: 1109–1117

Trentham, D. E,, Dynesius-Trentham, R. A., Orav, E. J., Combitchi, D., Lorenzo, C., Sewell, K. L., Hafler, D. A., Weiner, H. L. (1993) Effects of oral administration of type II collagen on rheumatoid arthritis. Science 261: 1727–1730

van Eden, W., Holoshitz, J., Vevo, Z., Frenkel, A., Klajman, A., Cohen, I. R. (1985) Arthritis induced by a T-lymphocyte clone that responds to *Mycobacterium tuberculosis* and to cartilage proteoglycans. Proc. Natl. Acad. Sci. USA 82: 5117–5120

van Vollenhoven, R. F., Soriano, A., McCarthy, P. E., Schwartz, R. L., Garbrecht, F. C., Thorbecke, J., Siskind, G. W. (1988) The role of immunity to cartilage proteoglycan in adjuvant arthritis. Intravenous injection of bovine proteoglycan enhances adjuvant arthritis. J. Immunol. 141: 1168–1173

Well, H. (1911) Studies on the chemistry of anaphylaxis. III. Experiments with isolated proteins, especially those of hen's egg. J. Infect. Dis. 9: 147–151

Wolf, J. L., Bye, W. A. (1984) The membranous epithelial (M) cell and the mucosal immune system. Annu. Rev. Med. 35: 95–112

Wood, F. D., Pearson, C. M, Tanaka, A. (1969) Capacity of mycobacterial wax D and its subfractions to induce adjuvant arthritis in rats. Int. Arch. Allergy Appl. Immunol. 35: 456–467