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Clinical Studies

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EFFECTS OF ORALLY ADMINISTERED UNDENATURED TYPE II COLLAGEN AGAINST ARTHRITIC INFLAMMATORY DISEASES: A MECHANISTIC EXPLORATION

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Summary: *Arthritis afflicts approximately 43 million Americans or approximately 16.6% of the US population. The two most common and best known types of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA). A significant amount of scientific research has been done in attempts to explain what initiates forms of arthritis, how it is promoted and perpetuated and how to effectively intervene in the disease process and promote cartilage remodeling. Current pharmacological strategies mainly address immune suppression and antiinflammatory mechanisms and have had limited success. Recent research provides evidence that alterations in the three-dimensional configuration of glycoproteins are responsible for the recognition/response signaling that catalyzes T-cell attack. Oral administration of autoantigens has been shown to suppress a variety of experimentally induced autoimmune pathologies, including antigen-induced RA. The interaction between gut-associated lymphoid tissue in the duodenum and epitopes of orally administered undenatured type II collagen facilitates oral tolerance to the antigen and stems systemic T-cell attack on joint cartilage. Previous studies have shown that small doses of orally administered undenatured type II chicken collagen effectively deactivate killer T-cell attack. A novel glycosylated undenatured type II collagen material (UC-II) was developed to preserve biological activity. The presence of active epitopes in the UC-II collagen is confirmed by an enzyme-linked immunosorbent assay test and distinguishes this form from hydrolyzed or denatured collagen. Oral intake of small amounts of glycosylated UC-II presents active epitopes, with the correct three-dimensional structures, to Peyer's patches, which influences the signaling required for the development of immune tolerance. UC-II has demonstrated*

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the ability to induce tolerance, effectively reducing joint pain and swelling in RA subjects. A pilot study was conducted for 42 days to evaluate the efficacy of UC-II (10 mg/day) in five female subjects (58-78 years) suffering from significant joint pain. Significant pain reduction including morning stiffness, stiffness following periods of rest, pain that worsens with use of the affected joint and loss of joint range of motion and function was observed. Thus, UC-II may serve as a novel therapeutic tool in joint inflammatory conditions and symptoms of OA and RA.

Introduction

Arthritis represents a group of debilitating diseases of the joints, bones, tendons, muscles and eventually organs. It afflicts approximately 43 million Americans, imposing a cost in excess of $65 billion annually (1). The two most common types are osteoarthritis (OA) and rheumatoid arthritis (RA), traditionally defined as age-related "wear-and-tear" arthritis and "autoimmune" arthritis, respectively (1, 2). However, inflammatory response has been identified to be a common mediator in both types of arthritis (1, 2).

Understanding of RA pathogenesis has changed over the years. RA is characterized by attack of killer T-cells on type II joint collagen, which results in damage to cartilage, joint swelling, pain and inflammation (3-6). The body's attempts to remodel joint cartilage are outpaced by immune mediated attack on and degradation of joint cartilage (3-6). Collectively, these events have been characterized as an out-of-control autoimmune response (3). Extensive research has explored the multifaceted dynamics of recognition, response and compensatory homeostatic mechanisms in an effort to understand, manage and maintain immune competence. Research in transgenic mice points to the possibility that B-lymphocytes and immunoglobulins outside the joint indirectly provoke RA pathogenesis *via* a self-reactive T-cell receptor in the joint (7). However, our understanding of autoimmunity still presents unresolved challenges that may require a paradigm shift in research for the development of effective and safe therapies.

It has been proposed that mechanisms involved in host defense, protection and maintenance of self-integrity are counteracting forces in which tolerance mechanisms efficiently suppress immune attack on self to a required threshold. An evolutionary perspective alleges that a tendency toward autoimmune malfunction should theoretically be higher during years when young immune systems are aggressively protecting the reproductive potential of the host. Misrecognition of self would be a predictable deficiency of the system (8). Autoimmune disorders are less prevalent in the young, increasing with advancing age and decline of reproductive potential. Indeed, there is a clear relationship between advancing age and an increased incidence of arthritis. To explain this, one rationale theorizes that RA disease must result from a deteriorating function of the immune system, which provides ideal conditions for a breakdown in self-tolerance (8). Decreased recognition and up-regulated self-attack is a logical conclusion consistent with an age-related decline in immune efficiency (9). However, explanations regarding "autoreactivity" of the immune system in RA disease favor an emphasis on functional flaws in surveillance, recognition and response (and their symptomatic manifestations) rather than the possibility that structural flaws in immune system complexes, and possi-

bly the target tissues, may be etiological catalysts. Recent strategies for therapeutic management of RA, therefore, focus on methods of inhibiting symptom manifestation to reduce the severity of the end-stage of this disease (10).

Etiological and therapeutic research faces the challenge of explaining how arthritic processes originate and progress (2). Most of the past and current work on rheumatoid diseases examine strategies to intervene or halt "out of control" immunologic and/or inflammatory events associated with autoimmune disease (10). The traditional paradigm proposes that RA is an immunological disorder for an as yet unidentified arthrogenic antigen. Various immunological factors are involved, such as CD4-inducer lymphocytes, CD4 cells, macrophages, neutrophils and tumor necrosis factor- (9, 10). This conventional view has procuded pharmacological therapies that favor manipulation of cyclooxygenase-2 events and immune suppression, with less than ideal results. Almost all of the biomolecules responsible for innate and adaptive immune response are glycoproteins (11). However, little attention is directed at the possibility that impaired glycosylation affects the configuration of glycoproteins, including IgG and type II collagen. These may alter recognition and response signaling during immune surveillance, inciting attack on the body's own joint collagen (11-19).

This view suggests that the term hyperreactive "immune abnormality" may be a misnomer for RA, as the immune system is behaving appropriately against host tissues ultimately identified as foreign pathogenic antigens (10). Altered glycosylation could produce a number of identification errors responsible for up-regulating self-attack. Among the possibilities are misidentification of type II joint collagen as antigenic by aberrant IgG, possible binding of hypogalactosylated IgG with certain rheumatoid factors leading to significant levels of immune complexes characteristic of RA and/or appropriate glycomic identification markers may be missing from the joint collagen and immune complexes. This perspective provides insight into how the immune system incurs a loss of self-tolerance and explores the possibility of flaws in glycosylation/galactosylation. This phenomenon is at the root of impaired immunological recognition and response activities for the hyper-autoreactive immune self-destruction of joint collagen in the pathogenesis of RA (19, 20). Hence, alterations in glycosylation/ galactosylation are hallmark characteristics of RA. This also provides a possible explanation as to who orally ingested native type II collagen produces tolerance, down-regulating autoimmune aggression (3, 4).

Impaired galactosylation affects glycoprotein synthesis, altering the requisite three-dimensional conformations of glycoproteins such as type II collagen and IgG, producing the loss of self-recognition. Lang and Yeaman (20) demonstrated that removal of carbohydrate moieties from antigens resulted in a loss of antibody binding. In RA patients, decreased levels of β1-4 galactosyltransferase activity in peripheral blood B- and T-lymphocytes correlates with the decreased galactosylation of serum IgG (13).

Immunoglobulins are by definition glycoprotein molecules produced by plasma cells in response to an immunogen, which function as antibodies (11). In RA, immune complexes that consist exclusively of immunoglobulin are present, indicating a role as both antibody and antigen. Both cartilage and immune system complexes are, for the most part, made of glycoprotein structures in which glycoprotein synthesis requires the necessary substrate and competent glycosylation (16). Impaired glycosylation/galactosylation intersects at a number of junctures contributing to the initiation, promotion and progression stages of RA (11-19).

Comparisons of the *N*-glycosylated pattern of serum IgG isolated from healthy individuals with that of RA patients demonstrates that differences ob-

served in RA patients are due to changes in the relative extent of glycosylation compared with normal individuals. In RA, an increased number of oligosaccharide structures lack the terminal galactose residue (19). This suggests that RA may be a glycosylation disease, reflecting changes in the intracellular processing, or post-secretory degradation of *N*-linked oligosaccharides (12, 19). Other research has reported a decrease in galactose residues in the oligosaccharide chains of the serum IgG of RA patients, which was presumed to affect the three-dimensional structure of the CH_2 domain. Galactose- depleted IgG reduced Clq binding and Fc receptor binding, which implies an important biological function of the glyconutrient moiety of IgG (16). Rademacher *et al.* (17) clearly demonstrated that galactose-deficient IgG glycoforms are directly associated with pathogenicity in collagen-induced rheumatoid arthritis in mice. Nonpathogenic autoantibodies were made pathogenic by altering their glycosylation state (17).

Immunization with undenatured type II collagen (antigen) has been shown to induce arthritis (21). However, orally ingested undenatured native antigens interact with gut-associated lymph tissue (GALT), resulting in an entirely opposite effect. Oral tolerization, using small doses of glycosylated undenatured type II collagen (UC-II), has demonstrated its effectiveness in turning off T-cell attack on type II joint collagen, inducing immunological hyporesponsiveness, and reducing pain and inflammation (3-6). In contrast, while denatured collagen may provide a nutritional source of substrate for joint cartilage synthesis, research demonstrates that it does not induce immunological hyporesponsiveness and has not demonstrated an effect on reducing pain and inflammation (6). Although the same amino acids are present in both forms, the tertiary and quaternary structures in the denatured form may be completely destroyed and the galactose moiety is degraded (Fig. 1), not allowing epitope recognition in the Peyer's patch (3, 10, 22). Furthermore, the hydrolyzed or denatured form may be pharmacologically ineffective because of the loss of conformation. Interestingly, the effects of oral tolerance do not appear to be confined to RA diseases alone, but confer appreciable benefits in some cases of OA as well. A pilot

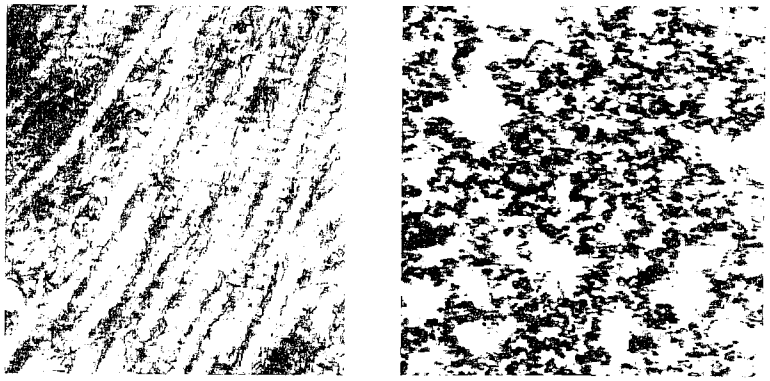

Fig. 1 Electron micrograph (magnification x 50,000) of undenatured type II collagen vs. denatured type II collagen. Undenatured type II collagen (on left) shows intact tertiary and quaternary glycoprotein integrity allowing for epitope recognition and hyporesponsive immune stimulation. Denatured type II collagen (on right) contains no tertiary and quaternary glycoprotein integrity.

study provides preliminary evidence that 10 mg/day of a commercial enzyme-linked immunosorbent assay (ELISA) verified undenatured glycosylated type II collagen (UC-II InterHealth Nutraceuticals Incorporated, Benicia, CA, USA) administered orally reduced sensory pain by 26% in four out of five women, aged 58-78 years old, for 42 days. Two of the women were previously diagnosed with OA and the remaining three exhibited similar symptoms but had no clinical diagnosis. There were no adverse effects associated with the intake of UC-II (Table I).

Peyer's patches are relatively large aggregates of lymph tissue located in the GALT of the small intestine (10, 22). The overlying "dome" epithelium contains large numbers of intraepithelial lymphocytes. Some of the epithelial cells have complex microfolds in their surfaces, known as M-cells. M-cells are important in the transfer of antigen from the gut lumen to the Peyer's patch (10). Peyer's patches then facilitate the generation of an immune response within the mucosa. An antigen in the Peyer's patch stimulates B-cell precursors and memory cells (10). Cells pass to the mesenteric lymph nodes where the immune response, if needed, is amplified. Activated lymphocytes pass into the blood stream *via* the thoracic duct. Oral tolerance occurs only after the correct three-dimensional conformation of UC-II antigen is identified as nonpathogenic (10, 22).

Materials and methods

Chemicals. Pepsin (Catalog number I.U.B. 3.4.23.1) was purchased from Worthington Biochemical Corporation (Freehold, NJ, USA). Unless otherwise stated, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

UC-II. UC-II was obtained from InterHealth Nutraceuticals. The presence of glycosylated "active" epitopes in the UC-II collagen matrix was confirmed by a validated ELISA test. Furthermore, electron microscopic analysis of UC-II was conducted to demonstrate the conformational integrity of the triple helical structure.

For electron microscopic analysis, a small amount of UC-II powder was fixed with Karnovsky fixative for 2 h, rinsed with cacodylate buffer for 20 min, placed in 1% osmium tetroxide for 2 h, rinsed with distilled water for 1 min and placed overnight in 0.5% uranyl acetate. The sample was then dried using ethanol and placed into propylene oxide for 30 min and finally placed in 50:50 propylene oxide:SPURR (embedding material) for 2 h and then into 100% SPURR overnight. It was then placed into a 70 °F oven overnight. A section was taken using ultra microtome, stained with uranyl acetate for 4 min, rinsed with distilled water, stained with lead citrate for

Table I *Measurement of pain level following a 42-day study of oral administration of undenatured type II collagen (UC-II)*

Subject #	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Reduction in Pain (%)
1	3	3	3	3	3	3	3	0
2	5	5	5	5	5	2	2	22
3	5	5	4	4	3	3	5	22
4	6	6	5	5	3	2	2	22
5	7	8	5	5	4	3	1	34

Administered dose: A single, daily oral dose of 10 mg glycosylated undenatured type II collagen (UC-II). Pain index: 10 = unbearable, 1 = tolerable.

2 min and rinsed again with distilled water and dried. The transmission electron microscope procedure was conducted in an EM JEOL 100CX (Peabody, MA, USA). An electron micrograph of undenatured type II collagen *vs.* denatured type II collagen is shown in Fig. 1. Undenatured type II collagen (on left) shows intact tertiary and quaternary glycoprotein integrity allowing for epitope recognition and hyporesponsive immune stimulation. Denatured type II collagen (on right) contains no tertiary or quaternary glycoprotein integrity. Epitopes of healthy undenatured type II collagen contain the correct composition and structural conformation of galactose-dependent glycoprotein, as evidenced by ELISA analysis (Fig. 2).

Time-dose measurements of UC-II activity in simulated human gastric fluid. Five samples of UC-II were analyzed for collagen activity *via* ELISA analysis. Samples were digested in pepsin, simulating an artificial stomach. The pepsin solution was made using 995 ml distilled water, 3.73 g KCl, 4 g HCl and 30 mg pepsin. Five collagen samples of 14.7 g each were incubated individually for 0, 15, 30, 60 and 90 min in 100 ml pepsin solution at 32 °C and pH 2.0. The digestion process was stopped by increasing the pH to 6.0 using 0.5 M NaOH solution. Both the solid material (insoluble collagen) and the supernatant (soluble collagen) were collected and analyzed for native type II collagen using a commercially available Capture ELISA kit supplied by Chondrex LLC (Redmond, WA, USA). The quantity of UC-II (mg%) was determined in both supernatant soluble type II collagen and insoluble type II collagen following incubation for 0, 15, 30, 60 and 90 min at 32 °C and pH 2.0.

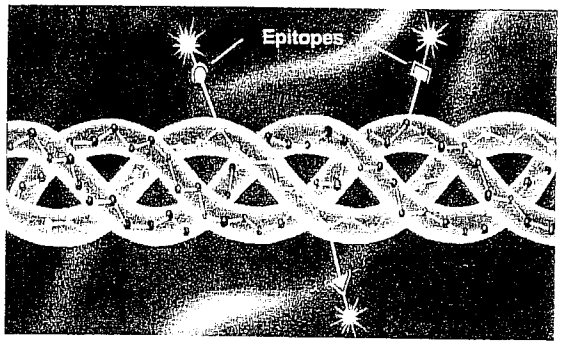

Fig. 2 Undenatured type II collagen triple helix molecule exhibiting epitope positions.

Pilot study to evaluate the efficacy of UC-II in human subjects. An open label pilot study was performed in five human subjects (women aged 58-78 years) suffering from significant joint pain, using a commercial ELISA-verified undenatured type II collagen (UC-II, InterHealth Nutraceuticals). To be eligible, patients had to meet the American College of Rheumatology criteria. Patients were excluded from the study if they had myocardial insufficiency, renal insufficiency (serum creatine > 2.0 mg/dl), disturbance of liver function, alkaline phosphatase > 300 units/liter, serum glutamic oxaloacetic transaminase > 50 units/liter, or bilirubin > 1.5 mg/dl), malignancy or a considerably reduced general state of health as determined by the physician. The five subjects enrolled in this study presented a history of osteoarthritis more than rheumatoid symptomology. These subjects reported early morning stiffness, stiffness following periods of rest, pain that worsened with use of the affected joint and loss of joint range of motion and function. Weather changes from warm to cold or dry to moist were also reported as pain-enhancing factors. All patients were required to sign an informed patient consent form prior to participation. The subjects were also given a questionnaire with detail protocol procedures, possible risks and benefits, *etc.* Two of the five subjects who suffered from osteoarthritis symptoms were clinically diagnosed 3 years prior to participation in this study. The remaining three subjects reported similar symptomology. Measurements included weekly diary-format

observations and qualitative feedback. Each subject received a single oral daily dose of 10 mg UC-II on an empty stomach prior to bedtime for 42 consecutive days. Each subject was asked to rate their respective pain level on a scale of 1-10, with a score of 10 representing "unbearable" and a score of 1 denoting "tolerable," prior to participation and immediately following completion of 7 days of treatment.

Results

Time-dose measurements of UC-II activity in gastric fluid. Following ingestion, the UC-II glycoprotein encounters hydrochloric acid and pepsin. Dose- and time-dependent studies were conducted to determine whether these monomers were still in the triple helical form, which we confirmed by ELISA assay. Figure 3 demonstrates the time-dose measurements of UC-II activity in simulated human gastric fluid at 32 °C and pH 2.0. Figure 3 clearly exhibits the UC-II activity in supernatant soluble type II collagen and insoluble type II collagen over a period of time (0-90 min). Thus, these results demonstrate that following incubation of UC-II for 90 min, approximately 50% of soluble UC-II is available to the epitopes.

Pilot study to evaluate the efficacy of UC-II in human subjects. An open label pilot study was conducted in five female subjects (aged 58-78 years) demonstrating the symptoms of significant joint pain. These subjects received a single oral daily dose of 10 mg UC-II on an empty stomach prior to bedtime for 42 consecutive days. All subjects rated their respective pain level on a scale of 1-10 (a score of 10 representing "unbearable" descending to a score of 1 denoting "tolerable"). The subjects rated their pain level before trial dose application and during treatment once every 7 days. Measurement of pain level in these human subjects following 42-day supplementation of UC-II is shown in Table I. Subject 1 perceived no reduction in her pain status throughout the open label trial. Subject 2 perceived a reduction in pain during the sixth week of the study, while under these same conditions Subjects 3, 4 and 5 reported a reduction in their pain level during the third week of treatment. Thus, a trial dose of 10 mg UC-II was associated with a ~26% reduction in perceived pain as indicated by four of the five subjects (22%, 22%, 22%, 34%; Table I). Furthermore, no side effects were associated with UC-II treatment. In essence, treatment with a daily oral dose of 10 mg UC-II was well tolerated and produced a significant reduction in joint pain symptoms.

Discussion

Epitope recognition. Epitopes (antigenic determinants) are structural components of an antigen molecule responsible for its specific interaction with T-cell antibody molecules elicited by the same or related antigen (23). Epitopes of healthy undenatured type II collagen contain the correct composition and structural conformation of galactose-dependent glycoprotein, as evidenced by ELISA analysis (24) (Fig. 2). A novel glycosylated undenatured type II collagen material (UC-II) was developed to preserve biological activity. The presence of glycosylated "active" epitopes in the UC-II collagen matrix is confirmed by a validated ELISA test and distinguishes this form from hydrolyzed, denatured agalactosylated collagen (25). Oral intake of 10 mg of this form of UC-II presents active epitopes, consisting of conformationally correct three-dimensional glycosylated structures, to Peyer's patches in the GALT (22, 26). Following ingestion, UC-II collagen glycoprotein encounters hydrochloric acid and pepsin. Dose- and time-dependent studies show these monomers are still in their triple helical form (Fig. 3) and travel down to the

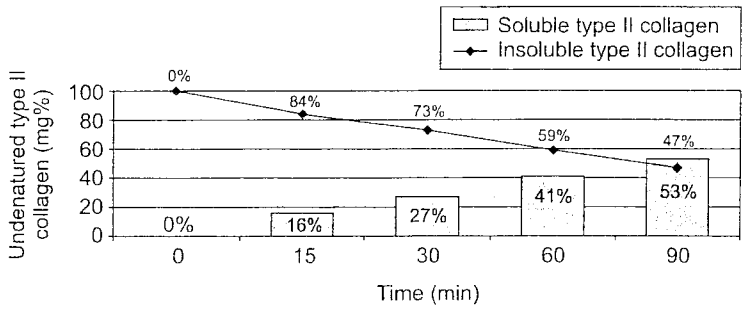

Fig. 3 Time-dose measurements of undenatured type II collagen activity in gastric fluid. Enzyme-linked immunosorbent measurements of undenatured type II collagen epitopes.

Peyer's patches, to which they bind. Pepsin does not break down the triple helical configuration of these monomers due to biochemical limitations, so the active sites always remain intact, which is confirmed by ELISA analysis. Pepsin will not cleave bonds containing the amino acids valine, alanine or glycine (27). The amino acid composition of native type II collagen is heavily distributed with glycine (28, 29). This glycine-rich sequence ensures that pepsin will not cleave the native collagen configuration (27). During digestion, the intact collagen fibril (a combination of collagen monomers, sugars and telopeptides) breaks down into monomeric collagen peptides (smaller glucopeptide units), exposing additional epitopes (30). On the other hand, the telopeptides bound to collagen molecules are susceptible to pepsin and get cleaved in the gut during digestion (31). This allows the collagen triple helix formation to loosen slightly, exposing additional active epitopes of the collagen glycoprotein, resulting in greater binding with and recognition by the Peyer's patches (32). These epitopes positively influence the immunoregulatory response signaling required for the development of tolerance (10, 32).

Properly glycosylated epitopes did not trigger T-cell proliferation, as did modified hybrid epitopes (21). Furthermore, Kim *et al.* (33) demonstrated that a single oral administration of poly(lactic-co-glycolic acid) (PLGA) nanoparticles induced tolerance against collagen II-induced arthritis in mice. Particles of PLGA were evident in the Peyer's patches of animals for 14 days from original feeding (33). Hyporesponsiveness results when epitopes of ingested UC-II collagen interact with the Peyer's patches in the lymphoid tissues of the duodenum, triggering the complex series of immunologic events that, in the case of RA, down-regulate the body's attack on its own type II joint collagen. This research demonstrated that PLGA was well tolerated against collagen II-induced arthritis. These active epitopes meet conformational specifications of the three-dimensional glycoprotein structures required by immune surveillance to signal approval and tolerance. Antigen epitope glycosylation has been shown to play an important role in T-cell recognition and B-cell responsiveness (21, 34, 35). This recognition and approval effectively turns off the up-regulated immune attack by reducing T-cell mediated inflammation, pain and swelling. UC-II has demonstrated its ability to induce tolerance, effectively reducing joint pain and swelling in RA subjects (3-6).

The science of glycobiology is rapidly expanding, uncapping enormous research opportunities and promising therapeutic tools (11). It provides new insights into disease initiation, promotion and progression, especially regarding autoimmune diseases, such as RA (12). A preponderance of the evidence suggests that all autoimmune diseases can be traced back to errors at some juncture of bioidentification, recognition and response signaling. Proper glycosylation is required for glycoconjugation, glycomolecular interconversions, biotransformations, and glycoprotein and glycolipid synthesis (11, 12).

In RA, impaired galactosylation alters the requisite three-dimensional conformations of glycoproteins, including certain immune factors, such as IgG and possibly even type II collagen, producing the loss of self-identity (12). Alterations in glycosylation and of galactosyl structures are hallmark characteristics of RA. This loss of self-identification alters recognition and response signaling during immune surveillance, inciting attack on the body's own joint collagen (13, 18).

Other autoimmune disorders have also been associated with faulty glycosylation (12, 17). This implies that certain autoimmune diseases may result when naturally occurring biomolecules are identified as foreign pathogenic antigens, due to their altered composition and structural conformation. As a result, appropriate immunological alarms are generated and aggressive defense tactics are employed against the host's own tissues (15).

Recently, safe and effective alternatives to traditional models of disease management have been used in RA (36). Oral administration of autoantigens has been shown to suppress a variety of experimentally induced autoimmune diseases, including antigen-induced RA (3-6, 33). As our understanding of glycobiochemistry increases, explanations regarding the reasons for these benefits emerge. Previous studies have shown that small doses of orally administered undenatured type II collagen effectively deactivate killer T-cell attack on type II joint collagen in humans (3, 22). Our pilot study exhibited the efficacy of UC-II (10 mg/day) in effectively reducing joint pain and swelling in human subjects without any adverse effects. UC-II contains conformationally correct "active" epitopes required to interact with Peyer's patches in the GALT and terminate antigenic signaling of a pathogenic nature, characteristic of RA (10). This approach provides new insights into the etiology of autoimmune inflammatory diseases and their amelioration with safe and effective treatments.

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References

(1) Trentham D.E., Halpner A.D., Trentham R.E., et al. *Use of undenatured type II collagen in the treatment of rheumatoid arthritis* Clin. Prac. Alter. Med., 2, 254, 2001.

(2) Helmick C.G., Lawrence R.C., Pollard R.A., et al. *Arthritis and other rheumatic conditions: Who is affected now, will be affected later?* Arthritis Care Res., 8, 203, 1995.

(3) Trentham D.E., Dynesius-Trentham R.A., Orav E.J., et al. *Effects of oral administration of type II collagen on rheumatoid arthritis.* Science, **261**, 1727, 1993.

(4) Barnett M.L., Combitchi D., Trentham D.E. *A pilot trial of oral type II collagen in the treatment of juvenile rheumatoid arthritis.* Arthritis Rheum., **39**, 623, 1996.

(5) Barnett M.L., Kremer J.M., St Clair E.W., et al. *Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo-controlled trial.* Arthritis Rheum., 41, 290, 1998.

(6) Nagler-Anderson C., Bober L.A., Robinson M.E., et al. *Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen.* Proc. Natl. Acad. Sci., 83, 7443, 1986.

(7) Benoist C., Mathis D. *A revival of the B cell paradigm for rheumatoid arthritis pathogenesis?* Arthritis Res., 2, 90, 2000.

(8) Weyand C.M., Brandes J.C., Schmidt D., et al. *Functional properties of CD4+ CD28- T cells in the aging immune system.* Mech. Ageing Dev., **102**, 131, 1998.

(9) Mowat M.A. *The regulation of immune responses to dietary protein antigens.* Immunol. Today, **8**, 93, 1987.

(10) Weiner H.L. *Oral tolerance: Immune mechanisms and treatment of autoimmune diseases.* Immunol. Today, **18**, 335, 1997.

(11) Rudd, P.M., Elliott T., Cresswell P., et al. *Glycosylation and the immune system.* Science, **291**, 2370, 2001.

(12) Parekh R.B., Dwek R.A., Sutton B.J., et al. *Association of rheumatoid arthritis and primary osteoarthritis with changes in glycosylation pattern of total serum IgG.* Nature, **316**, 452, 1985.

(13) Youings A., Chang S., Dwek R.A., et al. *Site-specific glycosylation of human immunoglobulin G is altered in four rheumatoid arthritis patients.* Biochem. J., **314**, 621, 1996.

(14) McCoy J.P., Chambers W.H. *Carbohydrates in functions of natural killer cells.* Glycobiology, **1**, 321, 1991.

(15) Tsuchiya N., Endo T., Matsuta K., et al. *Detection of glycosylation abnormality in rheumatoid IgG using N-acetylglucosamine-specific Psathyrella velutina lectin.* J. Immunol., **151**, 1137, 1993.

(16) Tsuchiya N., Endo T., Shiota M., et al. *Distribution of glycosylation abnormality among serum IgG subclasses from patients with rheumatoid arthritis.* Clin. Immunol. Immunopathol., **70**, 47, 1994.

(17) Rademacher T.W., Williams P., Dwek R.A. *Agalactosyl glycoforms of IgG autoantibodies are pathogenic.* Proc. Natl. Acad. Sci., **91**, 6123, 1994.

(18) Tsuchiya N., Endo T., Matsuta K, et al. *Effects of galactose depletion from oligosaccharide chains on immunological activities of human IgG.* J. Rheumatol., **16**, 285, 1989.

(19) Watson M., Rudd P.M., Bland M., et al. *Sugar printing rheumatic diseases: A potential method for disease differentiation using immunoglobulin G oligosaccharides.* Arthritis Rheum., **42**, 1682, 1999.

(20) Lang G.A., Yeaman G.R. *Autoantibodies in endometrioses sera recognize a Thomsen-Friedenreich-like carbohydrate antigen.* J. Autoimmun., **16**, 151, 2001.

(21) Corthay A., Backlund J., Broddefalk J., et al. *Epitope glycosylation plays a critical role for T cell recognition of type II collagen in collagen-induced arthritis.* Eur. J. Immunol., **28**, 2580, 1998.

(22) Sieper J., Kary S., Sorensen H. *Oral type II collagen treatment in early rheumatoid arthritis.* Athritis Rheum., **39**, 41, 1996.

(23) Burkhardt H., Koller T., Engstrom A., et al. *Epitope-specific recognition of type II collagen by rheumatoid arthritis antibodies is shared with recognition by antibodies that are arthritogenic in collagen-induced arthritis in the mouse.* Arthritis. Rheum., **46**, 2339, 2002.

(24) Fujii K., Tsuji M., Murota K. *An improved enzyme-linked immunosorbent assay of anti-collagen antibodies in human serum.* J. Immunol. Methods, **124**, 63, 1989.

(25) Williams P.J., Rademacher T.W. *Analysis of murine IgG isotype galactosylation in collagen-induced arthritis.* Scand. J. Immunol., **44**, 381, 1996.

(26) Terato K., Hasty K.A., Reife R.A. *Induction of arthritis with monoclonal antibodies to collagen.* J. Immunol., **148**, 2103, 1992.

(27) Ryle A.P. *The porcine pepsin and pepsinogens.* In: Perlmann G.E., Lerand L.(Eds.). "Methods in Enzymology." Vol. XIX, Academic Press, New York, 1970, p. 316.

(28) Miller E.J., Matukas V.J. *Chick cartilage collagen: A new type of alpha 1 chain not present in bone or skin of the species.* Proc. Natl. Acad. Sci., **64**, 1264, 1969.

(29) Trelstad R.L., Kang A.H., Igarashi S. *Isolation of two distinct collagens from chick cartilage.* Biochem., **9**, 4993, 1970.

(30) Herbage D., Bouillet J., Bernengo J.C. *Biochemical and physiochemical characterization of pepsin-solubilized type II collagen from bovine articular cartilage.* Biochem. J. **161**, 303, 1977.

(31) Ortolani F., Giordano M., Marchini M. *A model for type II collagen fibrils. Distinctive D-band patterns in native and reconstituted fibrils compared with sequence data for helix and telopeptide domains.* Biopolymers., **54**, 448, 2000.

(32) Meyer O. *Oral immunomodulation therapy in rheumatoid arthritis.* Joint Bone Spine., **67**, 384, 2000.

(33) Kim W.U., Lee W.K., Ryoo J.W. *Suppression of collagen-induced arthritis by single administration of poly(lactic-co-glycolic acid) nanoparticles entrapping type II collagen: A novel treatment strategy for induction of tolerance.* Arthritis Rheum., **46**, 1109, 2002.

(34) Schulte S., Unger C., Mo J.A. *Arthritis-related B cell epitopes in collagen II are conformation-dependent and sterically privileged in accessible sites of cartilage collagen fibrils.* J. Biol. Chem., **273**, 1551, 1998.

(35) Backlund J., Carlsen S., Hoger T. *Predominant selection of T cells specific for the glycosylated collagen type II epitope (263-270) in humanized transgenic mice and in rheumatoid arthritis.* Proc. Natl. Acad. Sci., **99**, 9960, 2002.

(36) Matteson E.L. *Current treatment strategies for rheumatoid arthritis.* Mayo Clin. Proc., **75**, 69, 2000.

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A PILOT TRIAL OF ORAL TYPE II COLLAGEN IN THE TREATMENT OF JUVENILE RHEUMATOID ARTHRITIS

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Objective. To evaluate the efficacy of oral chicken type II collagen (CCII) in the treatment of juvenile rheumatoid arthritis (JRA).

Methods. Ten patients with active JRA were treated with CCII for 12 weeks. Efficacy parameters, which included swollen and tender joint count and score, grip strength, 50-foot walking time, duration of morning stiffness, and patient and physician global scores of disease severity, were assessed monthly.

Results. All patients completed the full course of therapy. Eight patients had reductions in both swollen and tender joint counts after 3 months of CCII. The mean changes from baseline in swollen and tender joint counts for the 8 responders at the end of the study were −61% and −54%, respectively. Mean values for other efficacy parameters also showed improvement from baseline. There were no adverse events that were considered to be treatment related.

Conclusion. Oral CCII may be a safe and effective therapy for JRA, and its use in this disease warrants further investigation.

Juvenile rheumatoid arthritis (JRA) affects an estimated 65,000–70,000 children in the US (1). While it has been suggested that JRA has a better prognosis than adult rheumatoid arthritis (RA) (2), more recent data show that ~45% of children with JRA have active disease at 10-year followup (3).

Current treatment options for JRA are often unsatisfactory, because of both limited efficacy and concern about toxicity. These include antiinflammatory agents such as aspirin, naproxen, tolmetin, or ibuprofen, antimalarial agents, gold, and methotrexate, as well as physical therapy. In a minority of patients, rapidly progressive disease is refractory to these therapies and leads to permanent joint destruction with physical incapacitation. Systemic corticosteroids are relatively contraindicated in the treatment of JRA, except in patients with severe polyarthritis or severe systemic disease that has failed to respond to more conservative treatment. In addition to multiple other toxicities, growth suppression is a major deterrent to the use of steroids in the treatment of JRA. A multicenter study of D-penicillamine and hydroxychloroquine in the treatment of severe JRA showed that, when given in conjunction with a nonsteroidal antiinflammatory drug (NSAID), neither agent was superior to placebo (4). Methotrexate has been shown to be an effective treatment of refractory JRA (5), but parents and physicians alike remain concerned about possible long-term side effects. The toxic-to-therapeutic ratio of cytotoxic agents, such as cyclophosphamide, is even more narrow. Moreover, reports of malignancy either during or after therapy with immunosuppressive drugs have precluded their use in all but the most severely ill patients.

The evidence that sensitized T cells participate in provoking inflammation in RA and other rheumatic diseases (6) provides direction to the search for treatment modalities based on specific immunosuppression, which would be both highly effective and minimally toxic. The ability to induce antigen-specific peripheral immune tolerance by oral administration of antigens has been recognized for some time (7). It is presumed that the physiologic interaction of proteins with the gut immune system has evolved to prevent systemic immune responses to ingested proteins. Hypersensitivity reactions to food proteins are rare, and the mechanism of oral tolerance is based on this unique immunologic system. Given in low doses,

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orally administered antigens induce active immunosuppression, whereas high antigen doses lead to clonal anergy (8).

Oral administration of type II collagen has been shown to ameliorate arthritis in two animal models of RA, one induced by immunization with type II collagen (9,10) and the other induced by Freund's complete adjuvant (11). In addition, a placebo-controlled, phase II study in 60 adults with severe, active RA demonstrated significant ($P < 0.03$) improvement in tender and swollen joint counts after 3 months of treatment (12). A multicenter, double-blind, dose-ranging study of oral chicken type II collagen (CCII) in adult RA has recently been completed (Barnett ML et al: manuscript in preparation). The present open study of oral CCII in the treatment of JRA was undertaken based on these earlier results.

PATIENTS AND METHODS

A total of 10 patients with JRA were enrolled in the study. To be eligible, patients had to meet the American College of Rheumatology criteria for JRA (13). In addition, patients had to be between the ages of 8 and 14 years and had to have active arthritis, as defined by the presence of ≥3 swollen joints and ≥6 tender joints. Patients with any onset subtype were eligible provided that they had the required number of inflamed joints at the time of enrollment. Thus, a patient who had involvement of ≤4 joints within the first 6 months of disease (and who would therefore be classified as having pauciarticular onset) would nonetheless be eligible for enrollment in this study provided there were ≥3 swollen and ≥6 tender joints at the time of study entry. Patients were excluded if they were unable to discontinue treatment with disease-modifying antirheumatic drugs (DMARDs), if they had structural damage to the joints that was not considered to be amenable to physical rehabilitation if inflammation were to subside, or if they had serious concurrent medical problems.

During the course of the trial, patients were permitted to continue treatment with NSAIDs or low-dose corticosteroids (no more than the equivalent of 10 mg prednisone/day), provided that the doses remained stable during the treatment period. Increases in NSAID or prednisone dosage or initiation of any other antirheumatic therapy represented protocol violations. Patients were required to discontinue DMARDs at the start of the trial, with no mandated washout period.

Patients who met all entry criteria were enrolled and began treatment with CCII for a 3-month period. All patients and their parents were required to sign an informed consent form detailing protocol procedures, possible risks and benefits, etc. Treatment consisted of 100 μg/day of CCII for the first month and 500 μg/day thereafter. CCII was provided as a liquid suspension in 0.1*M* acetic acid at 4°C and added to cold orange juice immediately prior to ingestion. Doses and technique were the same as those used in the previous trial in adults (12). Patients were required to return for monthl visits, at which time safety and efficacy measurements wer obtained. Patients who exhibited an initial positive respons but subsequent worsening after the initial 3-month treatmer period were considered for further treatment with the stud medication, on a case-by-case basis.

Clinical efficacy was assessed by ascertaining painfi and swollen joint counts and joint scores according to th method of Weinblatt et al (14), evaluating a total of 5 diarthrodial joints for pain/tenderness and 52 joints fc swelling, duration of morning stiffness, grip strength, 50-foc walking time, and patient/parent and physician global score of disease activity at each visit. Laboratory data, includin complete blood cell count (CBC), erythrocyte sedimentatio rate (ESR), rheumatoid factor (RF) level, and serum Ig(antibodies to type II collagen (12), were recorded at baselin and after 3 months of therapy.

RESULTS

All 10 patients who enrolled and began stud medication completed the full 3 months of treatmen There were 5 girls and 5 boys, with a mean age of 10. years and a mean disease duration of 4.3 years. Th disease onset type was polyarticular in 3 patients pauciarticular in 3 patients, and systemic in 4 patients Four patients had previously been treated wit DMARDs, and 1 had been treated by his parents wit a variety of herbal medications. Patient 6 discontinue azathioprine 1 day prior to beginning therapy wit CCII, but no other patients were taking DMARDs a the time of enrollment. Six of the 10 patients receive concomitant stable doses of NSAIDs and/or low-dos prednisone during the study period (along with ace aminophen in 1); 1 patient continued to take acetamin ophen, and 3 patients took no concomitant medica tions for their arthritis. Eight of the 10 patients were i Steinbrocker functional class II (15) at study entry and the remaining 2 patients (patients 2 and 9) were i class III. HLA typing was not performed. Demc graphic and clinical features of the patients are pre sented in Table 1.

Eight patients had reductions in both swolle and tender joint counts after receiving CCII for months. The mean changes from baseline in swolle and tender joint counts for the 8 responders at the en of the study were −61% and −54%, respectively. Si patients had >33% reduction in both swollen an tender joint counts. Individual patient values for swol len and tender joint counts at baseline and after months of therapy are shown in Figure 1. The time t onset of response for the 10 patients was variable. I patient 1, almost all of the improvement was achieve within 1 month of the initiation of treatment, but th

Table 1. Demographic and clinical features of the patients with juvenile rheumatoid arthritis (JRA)*

Patient	Age/sex	Years of JRA	Onset subtype	Prior DMARDs	Concomitant medication
1	12/F	1	Poly	–	Napr. 250 mg twice a day
2	12/F	10	Systemic	MTX, HCQ	–
3	14/M	1.5	Pauci	†	–
4	9/F	0.5	Systemic	–	Napr. 250 mg twice a day, Pred. 2.5 mg/day
5	11/M	3	Pauci	SSZ	Ibu. 1,800 mg/day
6	11/M	7	Systemic	MTX, AZA, AUR	Pred. 2 mg/day
7	13/M	4	Pauci	MTX	Ibu. 800 mg/day
8	8/F	2	Poly	–	–
9	9/F	5.5	Systemic	–	Pred. 5 mg twice a day, acetaminophen
10	10/M	9	Poly	–	Acetaminophen

* Poly = polyarticular; Napr. = naproxen; MTX = methotrexate; HCQ = hydroxychloroquine; Pauci = pauciarticular; Pred. = prednisone; SSZ = sulfasalazine; Ibu. = ibuprofen; AZA = azathioprine; AUR = auranofin.
† Patient had been treated with herbal remedies prior to the initiation of chicken type II collagen treatment.

response occurred more slowly in other patients. On average for all 10 patients, the percentage of total improvement in swollen and tender joint counts achieved after only 1 month of treatment was 35% and 49%, respectively.

Swollen and tender joint scores decreased from baseline in 9 of the 10 patients. The mean reductions for all 10 subjects in swollen and tender joint scores after 3 months of therapy were 43% and 51%, respectively. These results are shown in Figure 2.

Mean values for morning stiffness and 50-foot walking time showed improvement from baseline. Clinical efficacy results for these parameters are presented in Figure 3. Although grip strength is not considered to be a reliable measure in children, mean values in right and left grip strength for the 10 patients did show a slight improvement from baseline to 3 months (data not shown). In addition, mean patient and physician global assessment scores also improved compared with baseline. One patient (patient 4) had total resolution of arthritis by the end of treatment and has subsequently been able to discontinue all medications with no return of symptoms during a 14-month followup period. No significant trends in any hematologic parameters, including CBC and ESR, were noted during the study. None of the patients tested positive for RF or collagen antibodies prior to or on completion of treatment.

CCII was well tolerated. Mild, transient skin rashes were noted in 4 patients during the study; in 3, the rash did not seem to be related to the study medication, and in no instance did the rash prompt

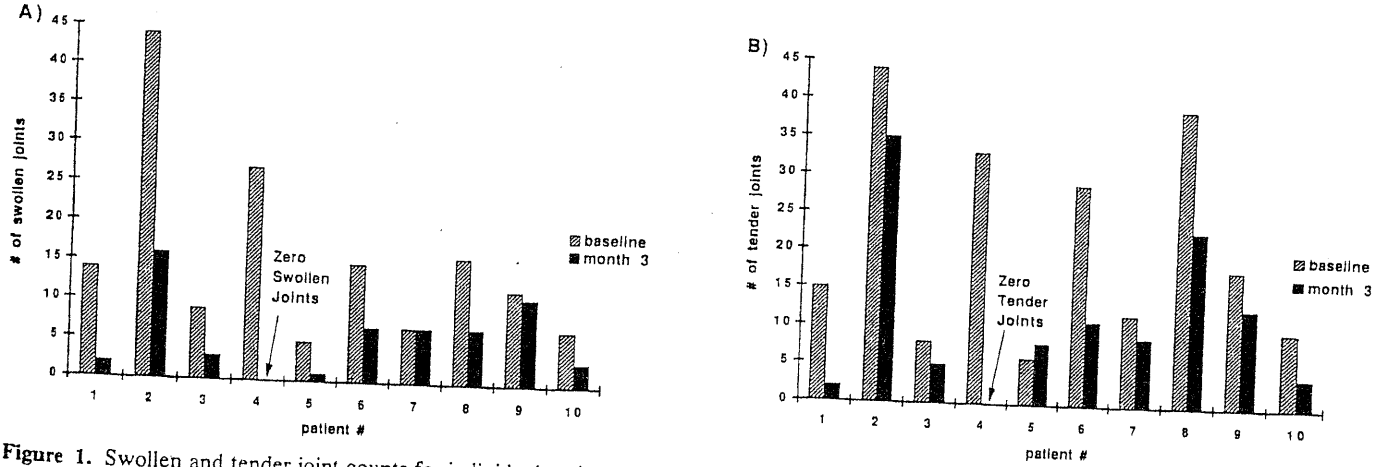

Figure 1. Swollen and tender joint counts for individual patients. The number of swollen (A) and tender (B) joints for each individual patient at baseline and after 3 months of treatment with chicken type II collagen is shown.

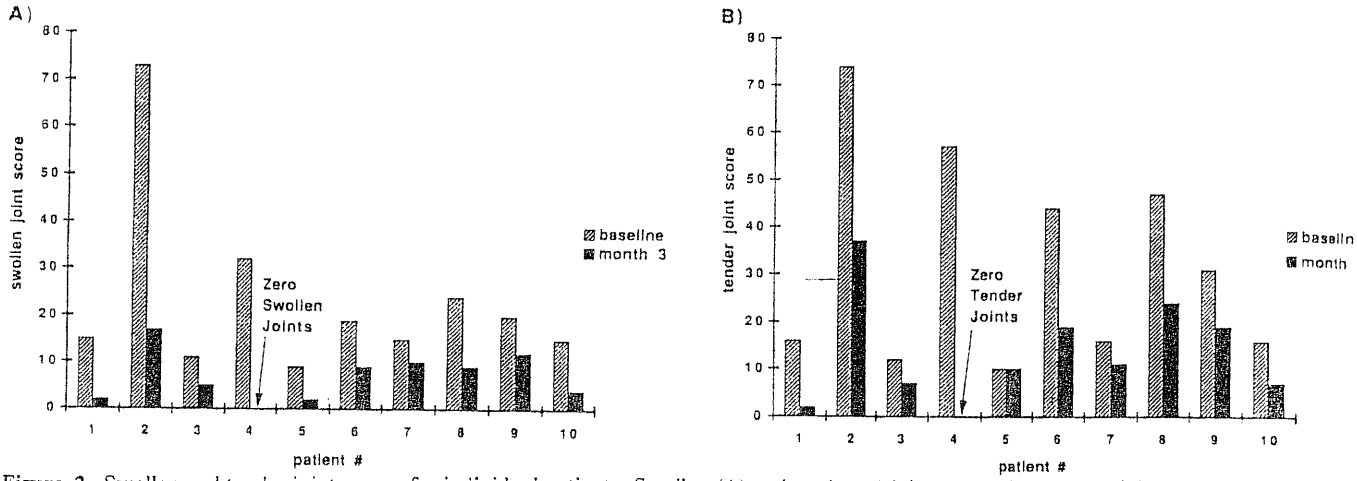

Figure 2. Swollen and tender joint scores for individual patients. Swollen (**A**) and tender (**B**) joint scores for each individual patient at baseline and after 3 months of treatment with chicken type II collagen are shown.

interruption of therapy. In 1 patient, an erythematous, pruritic rash was present on the legs at the time of study entry. This rash appeared to worsen during the first month of the trial, but it then resolved without specific therapy while the patient continued to take CCII. Two other patients reported transient erythematous rashes (not observed by the investigator) which were believed to be related to new soap or new laundry detergent. Facial flushing, which occurred 20 minutes after ingestion of CCII and lasted 1–2 hours, was noted by 1 patient during the initial 2 weeks of treatment, but subsequently resolved spontaneously.

Patient 6 had a history of chronic hepatitis C at the time of study entry. During the second month of the trial period, the findings on routine blood tests performed by his personal physician were notable for elevated transaminase levels. His only symptom a that time was an increase in fatigue. One week later when his transaminase levels were found to have risen further, he underwent a liver biopsy. This revealed mild chronic active hepatitis similar to that exhibited on a previous biopsy performed in 1991, and it was decided that his dosage of oral corticosteroids should be increased. Repeat liver function tests (LFTs) performed the day prior to the initiation of high-dose prednisone treatment demonstrated spontaneous improvement in his transaminase values to <50% of their peak levels, but this test result became available only after the patient had taken one 20-mg dose of prednisone. The patient discontinued high-dose prednisone after this single dose, and his LFT findings returned to normal within 1 week and subsequently remained

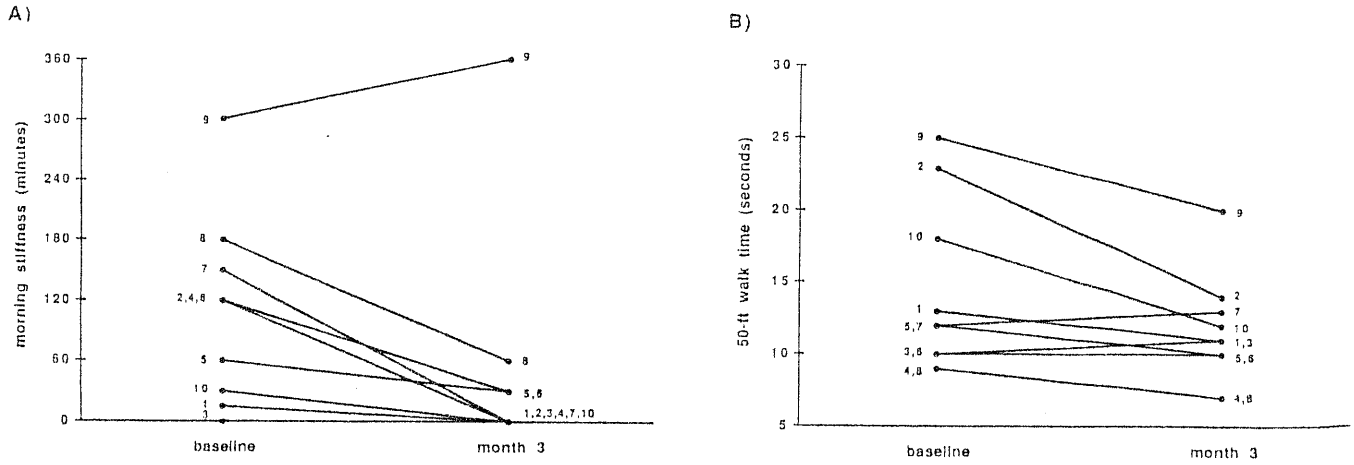

Figure 3. Secondary efficacy parameters (**A**, morning stiffness; **B**, 50-foot walking time) at baseline and after 3 months of treatment with chicken type II collagen. Individual patient numbers are shown on the graphs next to their respective plot markers.

normal for the duration of the study. At no time during this period was his CCII therapy interrupted, and this transient rise in LFT values was not believed to be related to the study medication. Of note, since the conclusion of the trial, the patient has had another similar episode of transient transaminitis while not taking CCII.

After conclusion of the study protocol, a second 3-month course of CCII was requested for and provided to 4 patients (patients 1, 2, 7, and 8). Patient 4 was examined 14 months after study completion, at which time it was confirmed that she remained completely free of any symptoms of arthritis with no medications, had no tender or swollen joints, and had normal laboratory values.

DISCUSSION

Oral tolerization is a well-recognized phenomenon in which the oral administration of antigen induces peripheral immune tolerance to the fed antigen (7). The utility of oral tolerization as a treatment modality for a variety of autoimmune diseases, including RA (12), multiple sclerosis (16), type I diabetes mellitus (17), and uveitis (18), is currently under active investigation. To date, no significant adverse events have been noted in any animal or human study of oral tolerance, and the simplicity and apparent safety of this form of treatment make it extremely appealing in these chronic, disabling diseases.

Based on results of animal studies, the mechanism responsible for oral tolerance varies depending on the dose of fed antigen, with low doses inducing active suppression and high doses resulting in clonal anergy (8). The regulatory cells that orchestrate active suppression act via the secretion of suppressive cytokines, such as transforming growth factor β and interleukin-4 (19). Experiments in animals support the notion of the generation of regulatory lymphocytes in Peyer's patches which subsequently migrate to mesenteric lymph nodes and spleen (20). Secretion of regulatory cytokines by these cells in vitro is dependent on antigen-specific stimulation with the fed antigen (21). Thus, it is presumed that active suppression of inflammation by these regulatory lymphocytes requires further migration of these cells to a local microenvironment, where the fed antigen is present.

Because the regulatory cells generated by oral tolerization are primed in an antigen-specific manner but suppress in a non–antigen-specific manner, they mediate "bystander suppression" when they encounter the fed autoantigen at other sites. This phenomenon of bystander suppression has been demonstrated in experimental autoimmune encephalomyelitis (EAE), a cell-mediated autoimmune disease that serves as an animal model for multiple sclerosis. EAE can be induced by immunization with myelin basic protein (MBP) or proteolipid protein (PLP). Oral administration of MBP has been shown to suppress both MBP- and PLP-induced EAE (22). Similarly, oral administration of type II collagen has been shown to ameliorate RA induced in animal models by immunization with either Freund's complete adjuvant (11), CCII (9,10), or methylated bovine serum albumin (23). Thus, it may not be necessary to identify the target autoantigen for a given disease. It is necessary only to orally administer a protein which is present at the site of inflammation and which is capable of inducing regulatory cells to secrete suppressive cytokines. These findings have important implications for the use of oral tolerance as a therapeutic approach for the treatment of T cell–mediated inflammatory autoimmune diseases in humans in which the inciting autoantigen is unknown or in which there is autoreactivity to multiple autoantigens in the target tissue.

Alternatively, a dominant pathway for oral tolerance may involve T cell anergization (24,25). In this case, the induction of oral tolerance would be presumed to result in disease suppression only when the fed antigen is also the target autoantigen for the disease under study. The demonstration of a sustained remission of arthritis in 1 of our 10 patients might arguably be more consistent with this latter view, based on the longevity of her response. However, this would imply that type II collagen was the disease-specific autoantigen in her case, and while collagen reactivity can be demonstrated in some patients with RA, it is unknown whether this is actually involved in the primary pathogenesis of the disease or merely reflects tissue degradation.

The present study demonstrates that oral CCII may be a safe and effective form of treatment for JRA. The most remarkable improvements in clinical parameters of arthritis were noted in patients 1 and 4, both of whom were girls with relatively recent onset of disease. Patient 1 had polyarticular onset, whereas patient 4 had systemic features of fever and rash in addition to polyarticular joint involvement at onset. Of note, of the 3 boys with pauciarticular onset of disease, 2 experienced minimal, if any, benefit from collagen (patients 5 and 7). As mentioned above, HLA typing was not performed, but it would be of interest

to know whether these patients were HLA–B27 positive. If this were the case, it might suggest that type II collagen is ineffective in the treatment of juvenile spondylarthropathies.

In an open-label study, one must always be concerned about the contribution of the placebo effect, and this may be even more true in a pediatric population. Therefore, conclusions regarding efficacy based on this pilot trial would be premature, but nonetheless, these preliminary data support the assertion that further study of oral CCII in the treatment of JRA is warranted. The observation that 1 patient achieved a complete remission of her arthritis is especially compelling in this regard and is similar to the experience observed in a minority of adults treated with CCII (12). More importantly, as pertains to this pilot study, oral CCII appears to be extremely well tolerated in this pediatric patient population. The only adverse event noted during the study that was believed to be related to the study medication was transient facial flushing, which occurred in 1 patient for ~2 weeks after collagen treatment was begun. The elevated transaminase levels noted in patient 6 during the second month of the study resolved without interruption of collagen therapy and were believed to be related to his underlying chronic hepatitis C. The combination of favorable safety data and promising clinical results in this pilot trial strongly indicate that there should be further studies of this novel therapeutic agent in the treatment of JRA.

REFERENCES

1. Singsen BH: Epidemiology of the rheumatic diseases of childhood. Rheum Dis Clin North Am 16:581–599, 1990
2. Hanson V, Kornreich H, Bernstein B, King KK, Singsen B: Prognosis of juvenile rheumatoid arthritis. Arthritis Rheum 20 (suppl 2):279–284, 1977
3. Wallace CA, Levinson JE: Juvenile rheumatoid arthritis: outcome and treatment for the 1990's. Rheum Dis Clin North Am 17:891–905, 1991
4. Brewer EJ, Giannini EH, Kuzmina N, Alekseev L: Penicillamine and hydroxychloroquine in the treatment of severe JRA: results of the USA-USSR double-blind, placebo-controlled trial. N Engl J Med 314:1269–1276, 1986
5. Giannini EH, Brewer EJ, Kuzmina N, Shaikov A, Maximov A, Vorontsov I, Fink CW, Newman AJ, Cassidy JT, Zemel LS: Low-dose methotrexate in resistant JRA: results of the USA-USSR double-blind, placebo-controlled trial. N Engl J Med 326:1043–1049, 1992
6. Sewell KL, Trentham DE: Pathogenesis of rheumatoid arthritis. Lancet 341:283–286, 1993
7. Mowat A: The regulation of the immune responses to dietary protein antigens. Immunol Today 8:93–98, 1987
8. Friedman A, Weiner HL: Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. Proc Natl Acad Sci U S A 91:6688–6692, 1994
9. Nagler-Anderson C, Bobber LA, Robinson ME, Siskind GW, Thorbecke GJ: Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen. Proc Natl Acad Sci U S A 83:7443–7446, 1986
10. Thompson HSG, Staines NA: Gastric administration of type II collagen delays the onset and severity of collagen-induced arthritis in rats. Clin Exp Immunol 64:581–586, 1986
11. Zhang ZY, Lee CS, Lider O, Weiner HL: Suppression of adjuvant arthritis in Lewis rats by oral administration of type II collagen. J Immunol 145:2489–2493, 1990
12. Trentham DE, Dynesius-Trentham RA, Orav EJ, Combitchi D, Lorenzo C, Sewell KL, Hafler DA, Weiner HL: Effects of oral administration of type II collagen on rheumatoid arthritis. Science 259:1321–1324, 1993
13. Brewer EJ Jr, Bass J, Baum J, Cassidy JT, Fink C, Jacobs J, Hanson V, Levinson JE, Schaller J, Stillman JS: Current proposed revision of JRA criteria. Arthritis Rheum 20 (suppl 2):195–199, 1977
14. Weinblatt ME, Coblyn JS, Fox DA, Fraser PA, Holdsworth DE, Glass DN, Trentham DE: Efficacy of low-dose methotrexate in rheumatoid arthritis. N Engl J Med 312:818–822, 1985
15. Steinbrocker O, Traeger CH, Batterman RC: Therapeutic criteria in rheumatoid arthritis. JAMA 140:659–662, 1949
16. Weiner HL, Mackin GA, Matsui M, Orav EJ, Khoury SJ, Dawson DM: Double-blind pilot trial or oral tolerization with myelin antigens in multiple sclerosis. Science 259:1321–1324, 1993
17. Zhang ZJ, Davidson L, Eisenbarth G, Weiner HL: Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. Proc Natl Acad Sci U S A 88:10252–10256, 1991
18. Nussenblatt RB, Caspi RR, Mahdi R, Chan CC, Roberge F, Lider O, Weiner HL: Inhibition of S-antigen induced experimental autoimmune uveoretinitis by oral induction of tolerance with S-antigen. J Immunol 144:1689–1695, 1990
19. Khoury SJ, Hancock WW, Weiner HL: Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor β, interleukin 4, and prostaglandin E expression in the brain. J Exp Med 176:1355–1364, 1992
20. Richman LK, Graeff AS, Yarchoan R, Strober W: Simultaneous induction of antigen-specific IgA helper T cells and IgG suppressor T cells in the murine Peyer's patch after protein feeding. J Immunol 126:2079–2083, 1981
21. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL: Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265:1237–11240, 1994
22. Al-Sabbagh A, Miller A, Santos LMB, Weiner HL: Antigen-driven tissue-specific suppression following oral tolerance: orally administered myelin basic protein suppresses proteolipid protein-induced experimental autoimmune encephalomyelitis in the SJL mouse. Eur J Immunol 24:2104–2109, 1994
23. Yoshino S, Quattrocchi E, Weiner HL: Suppression of antigen-induced arthritis in Lewis rats by oral administration of type II collagen. Arthritis Rheum 38:1092–1096, 1995
24. Whitacre CC, Gienapp IE, Orosz CG, Bitar DM: Oral tolerance in experimental autoimmune encephalomyelitis. III. Evidence for clonal anergy. J Immunol 147:2155–2163, 1991
25. Garside P, Steel M, Worthey EA, Satoskar A, Alexander J, Bluethmann H, Liew FY, Mowat AMcI: T helper 2 cells are subject to high dose oral tolerance and are not essential for its induction. J Immunol 154:5649–5655, 1995

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TREATMENT OF RHEUMATOID ARTHRITIS WITH ORAL TYPE II COLLAGEN

Results of a Multicenter, Double-Blind, Placebo-Controlled Trial

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Objective. **Oral administration of cartilage-derived type II collagen (CII) has been shown to ameliorate arthritis in animal models of joint inflammation, and preliminary studies have suggested that this novel therapy is clinically beneficial and safe in patients with rheumatoid arthritis (RA). The present study was undertaken to test the safety and efficacy of 4 different dosages of orally administered CII in patients with RA.**

Methods. **Two hundred seventy-four patients with active RA were enrolled at 6 different sites and randomized to receive placebo or 1 of 4 dosages (20, 100, 500, or 2,500 μg/day) of oral CII for 24 weeks. Efficacy parameters were assessed monthly. Cumulative response rates (percentage of patients meeting the criteria for response at any time during the study) were analyzed utilizing 3 sets of composite criteria: the Paulus criteria, the American College of Rheumatology criteria for improvement in RA, and a requirement for ≥30% reduction in both swollen and tender joint counts.**

Results. **Eighty-three percent of patients completed 24 weeks of treatment. Numeric trends in favor of the 20 μg/day treatment group were seen with all 3 cumulative composite measures. However, a statistically significant increase ($P = 0.035$) in response rate for the 20 μg/day group versus placebo was detected using only the Paulus criteria. The presence of serum antibodies to CII at baseline was significantly associated with an increased likelihood of responding to treatment. No treatment-related adverse events were detected. The efficacy seen with the lowest dosage is consistent with the findings of animal studies and with known mechanisms of oral tolerance in which lower doses of orally administered autoantigens preferentially induce disease-suppressing regulatory cells.**

Conclusion. **Positive effects were observed with CII at the lowest dosage tested, and the presence of serum antibodies to CII at baseline may predict response to therapy. No side effects were associated with this novel therapeutic agent. Further controlled studies are required to assess the efficacy of this treatment approach.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease that is characterized by pain, swelling, and stiffness of multiple joints. Chronic joint inflammation commonly results in progressive joint destruction, deformity, and loss of function. Current therapies for RA are often unsatisfactory, both because of inadequate efficacy and because of unacceptable toxic effects.

Evidence that autoreactive, sensitized T cells

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participate in sustaining the inflammation of RA (1) provides direction to the search for therapeutic strategies based on specific immunosuppressive actions that would be both highly effective and minimally toxic. The ability to induce antigen-specific peripheral immune tolerance by oral administration of antigens has been recognized for some time (2), and it is presumed that the physiologic interaction of proteins with the gut immune system has evolved to prevent systemic immune responses to ingested proteins. The possibility that autoimmune disease may be ameliorated by the induction of oral tolerance to disease-relevant autoantigens is currently under active investigation. It has been shown that oral administration of autoantigens suppresses a variety of experimental autoimmune diseases, including experimental allergic encephalomyelitis (EAE) (3,4), as well as collagen- (5,6), adjuvant- (7), and antigen- (8) induced arthritis. Preliminary results in humans have been encouraging, primarily in patients with multiple sclerosis using peroral administration of myelin (9) and in patients with RA using peroral administration of type II collagen (CII) (10,11).

Although the precise mechanisms of oral tolerance are not fully known, studies of EAE have shown, by immunohistologic analysis of brain tissue, that oral antigen delivery preferentially increases expression of the inhibitory cytokines transforming growth factor β (TGFβ) and interleukin-4 (IL-4), and decreases expression of the proinflammatory cytokines IL-1, IL-2, IL-6, IL-8, tumor necrosis factor α (TNFα), and interferon-γ (12). The net outcome is a lessening of brain inflammation. Given in low doses, orally administered antigens induce active immune suppression, whereas high antigen doses lead to clonal anergy (13) or deletion (14).

In addition to its positive effect in animal models of arthritis, oral administration of CII may benefit humans with RA. A placebo-controlled, phase II study involving 60 adults with severe, active RA demonstrated significant ($P < 0.03$) decreases in tender and swollen joint counts after 3 months of oral CII treatment (10). A 3-month open-label trial of CII in juvenile rheumatoid arthritis also was associated with clinical improvement in disease measures without significant toxicity (15). The present multicenter, double-blind, dose-ranging study of oral CII in adult RA was undertaken as a phase II trial to extend these earlier results and to investigate the relationship between dosage and clinical response.

PATIENTS AND METHODS

Patients. To be eligible for this trial, patients had to meet the 1987 American College of Rheumatology (ACR; formerly, the American Rheumatism Association) classification criteria for RA (16) and be between the ages of 18 and 80 years, with onset of RA after age 16. Patients were required to have at least 6 swollen and 9 tender joints, and duration of disease had to have been at least 6 months. Women of childbearing potential were required to have a negative pregnancy test result at baseline and to be using an effective contraceptive measure. Patients were required to discontinue treatment with disease-modifying antirheumatic drugs (DMARDs) before entering the study, with a variable washout period depending on the specific medication (at least 8 weeks for methotrexate, azathioprine, minocycline, cyclosporine, and cyclophosphamide, and at least 12 weeks for hydroxychloroquine, sulfasalazine, oral or intramuscular gold, and penicillamine).

Patients were excluded from the trial if they had previously been treated with oral CII or anti-TNF antibodies. Patients taking therapeutic doses of fish oil or plant oil ($\geq$2 gm/day), or shark cartilage or cartilage from another species were also excluded. Patients were not eligible if they had documented human immunodeficiency virus infection, a history of substance abuse within 1 year prior to study entry, a history of gastrointestinal disease which might affect collagen absorption and processing, serious intercurrent/underlying disease which would limit successful participation in the trial, or were classified in Steinbrocker functional class IV (17).

Study design. The study was a 6-center, double-blind, randomized, placebo-controlled trial comparing different dosages of CII and placebo in the treatment of RA. Upon meeting all entry criteria, patients were enrolled and were subsequently stratified by rheumatoid factor status. This was done to ensure that the treatment groups would be balanced with regard to rheumatoid factor positivity. At each of the 6 investigative sites, randomization of patients to the 5 treatment groups was accomplished in blocks of 5 within each stratum (positive versus negative for rheumatoid factor). Patients and investigators were blinded to the treatment regimens throughout the study.

Patients were allowed to remain on their nonsteroidal antiinflammatory drug (NSAID) and/or oral corticosteroid regimens provided that the steroid dosage was $\leq$10 mg of prednisone equivalent, and that neither the NSAID nor steroid dosage was changed during the study. Patients who requested analgesics for pain management were supplied these medications as needed during the period of study. Analgesic ingestion was recorded at each visit.

Patients were provided with a 1-month supply of masked study medication at the baseline visit and every 4 weeks thereafter. Liquid study medication was supplied in the form of patient kits containing each daily dose in individual polystyrene tubes. The study medication required refrigeration. Therefore, patients were given coolers to transport the medication home and were told to keep the kits in the refrigerator. Patients were instructed to empty the contents of one tube into a 4–6-ounce glass of cold orange juice 20 minutes prior to eating breakfast, and were required to consume the entire amount every morning.

Study medication. Native CII was prepared from the sternal cartilage of chickens by the method of Trentham et al (18). The CII was diluted in 0.1*M* acetic acid, and supplied as a single daily dose of 20 μg, 100 μg, 500 μg, or 2,500 μg. The

placebo was an equivalent volume of dilute acetic acid that was visually indistinguishable from the active drug.

Outcome measures. Clinical assessments of efficacy were made at baseline and repeated 2, 4, 8, 12, 16, 20, and 24 weeks later (except the Health Assessment Questionnaire [HAQ] [19], which was completed only at baseline and weeks 12 and 24). Fifty-four diarthrodial joints were examined for the presence or absence of tenderness, and 52 were examined for swelling (hips excluded). Joint counts for tenderness and swelling were the sum of the number of affected joints (20). These joints were also evaluated for the severity of both tenderness and swelling using a 4-point scale, in which 0 = none, 1 = mild, 2 = moderate, and 3 = severe. The sum of these determinations for all joints constituted a collective score for either tenderness or swelling (20). During the trial, a strong emphasis was placed on having all joint assessments for each patient done by the same evaluator.

Grip strengths were measured bilaterally using mercury-strain sphygmomanometers which were fitted with custom-made covers to assure a uniform circumference of the cuff. Fifty-foot walking times were measured in seconds. Physician and patient global assessments of disease activity were performed using a scale of 1–5, in which 1 = absent, 2 = mild, 3 = moderate, 4 = severe, and 5 = very severe. In addition, both the physicians and the patients rated progress from the previous visit, according to the categories much worse, worse, same, better, or much better (20). Patients were questioned about the duration of morning stiffness experienced on the day before each study visit. Functional class was assessed according to the Steinbrocker classification (17) at the screening visit and again at 24 weeks. Functional status was assessed at baseline and at weeks 12 and 24 using the HAQ.

Adverse events were monitored at every visit. Moreover, at baseline and at weeks 2, 4, 12, and 24, laboratory parameters were measured to assess safety. The erythrocyte sedimentation rate (ESR) was obtained by the Westergren method at baseline and at every visit thereafter, and rheumatoid factor positivity was determined at the screening visit and at week 24. Extra serum samples obtained at baseline, week 12, and week 24 were frozen and stored until the end of the study, for measurement of C-reactive protein (CRP) and IgA and IgG antibody titers to native chicken CII by enzyme-linked immunosorbent assay (ELISA). The ELISA was performed utilizing wells coated with purified CII, patients' serum, and horseradish peroxidase–linked goat anti-human IgA and IgG antibodies.

Patients who dropped out before completing 24 weeks of treatment were evaluated immediately prior to exiting the study. During this early-termination visit, safety and efficacy measurements were obtained.

Statistical analysis. Safety assessments were performed on all patients who consumed any masked study medication. Efficacy analyses were performed on the intent-to-treat population (all patients for whom we had at least 1 post-baseline measurement) as well as on the population of patients who completed the 24-week study. Balance within each treatment group with respect to patient characteristics at baseline was assessed, using Fisher's exact test for categorical variables and analysis of variance for continuous variables. Differences were considered statistically significant if the 2-sided P value was ≤ 0.05.

The primary efficacy end point was cumulative response, paralleling other recent studies of potential therapies for RA (21,22). Patients were said to be "responders" if they met the response criteria at any time during the study and were said to be "nonresponders" if they never showed a response to treatment during the study. This approach allowed each patient to be assessed just once, thus avoiding the criticisms of repeated significance testing and dropout effects, and was thought to be appropriate for a phase II trial which was primarily exploratory in nature. Response was analyzed with respect to 3 composite response-criteria sets. These were the Paulus criteria (23), the ACR criteria for improvement in RA (24), and the requirement for an improvement of $\geq$30% in both the tender and the swollen joint counts. The a priori contrasts planned for this study were to compare each active treatment group with the control group. The study had 80% power to detect a 25% difference in response rates between best dose and placebo (which was expected to have a response rate of 20%) for a 2-sided hypothesis test with 5% type I error. A logistic regression model enabled us to simultaneously compare response rates between each active treatment and placebo group while controlling for patient characteristics, without making multiple comparisons. The patient characteristics included in the model were age, sex, baseline tender and swollen joint counts, baseline rheumatoid factor status and ESR, Steinbrocker functional class at baseline, DMARD washout interval prior to baseline, baseline NSAID use, baseline corticosteroid use, duration of RA, the extent of analgesic use during the study, and study site. Interactions between each treatment level and each of the covariates were assessed.

Individual disease parameters that were analyzed as primary efficacy end points included tender and swollen joint counts, and physician and patient global assessments of disease severity. Safety was assessed by comparing the type and incidence of treatment-emergent events and laboratory abnormalities reported. Adverse events were classified using COSTART.

RESULTS

A total of 297 patients with active RA were screened for study eligibility, of whom 274 met the entrance criteria and were randomized to receive 1 of 4 doses of CII or matching placebo for a 24-week period. The characteristics of the enrolled patients are summarized in Table 1. There were no statistically significant differences between the treatment groups in demographic or disease characteristics. One patient in the 500 μg/day CII group dropped out prior to receiving any post-baseline efficacy assessments and was therefore not included in the intent-to-treat analysis.

A total of 228 patients (83%) completed the full 6-month treatment period. The frequency of dropouts was similar across all treatment groups. Of the 46 dropouts, 42 were due to inadequate therapeutic effect

Table 1. Patient characteristics at baseline, by treatment group*

Variable	Placebo (n = 57)	Oral type II collagen			
		20 μg/day (n = 54)	100 μg/day (n = 55)	500 μg/day (n = 53)	2,500 μg/day (n = 54)
Age, years	53.6 ± 12.1	53.5 ± 11.3	50.3 ± 10.9	49.4 ± 13.0	51.3 ± 14.1
Sex, % female	89	76	89	81	83
Disease duration, years	13.1 ± 9.6	12.8 ± 8.1	11.7 ± 8.4	9.8 ± 8.3	11.8 ± 10.9
RF positive, %	72	76	76	75	74
Steinbrocker functional class, %					
Class I	0	2	2	4	2
Class II	81	74	78	85	80
Class III	19	24	20	11	18
Westergren ESR, mm/hour	36 ± 22	41 ± 32	36 ± 27	35 ± 23	38 ± 26
NSAID use at entry, %	81	76	87	79	78
Oral corticosteroid use at entry, %	53	59	47	55	37
Previous DMARD use, %	89	87	91	96	81
No. of dropouts during study	12	11	5	7	10

* Except where otherwise indicated, values are the mean ± SD. There were no statistically significant differences in any feature between the treatment groups. RF = rheumatoid factor; ESR = erythrocyte sedimentation rate; NSAID = nonsteroidal antiinflammatory drug; DMARD = disease-modifying antirheumatic drug.

and 4 were due to adverse events (1 from the placebo group and 3 from the CII group). Although some of the patients took additional antiinflammatory medications during the trial, none were dropped from the analysis because of these protocol violations. Rules were developed before the analysis to address the use of protocol-excluded medical therapy. If a patient received an intraarticular corticosteroid injection, that joint was automatically assigned a grade of 3 (severe) for both tenderness and swelling at the next visit. This convention affected efficacy data for a single joint at 1 visit in 7 patients. Because of concern about possible systemic effects from an intraarticular steroid injection, a review of clinical efficacy data was undertaken to determine whether any of these 7 patients met the criteria for response only at the visit immediately following a joint injection. This was determined not to be the case for any of these 7 patients. If a patient took any antiinflammatory drug prohibited by the protocol for 2 of the 3 days prior to a visit, efficacy data from that visit were excluded from all analyses. The application of this rule did not result in the exclusion of any efficacy data.

Clinical response was assessed in the intent-to-treat population with respect to 3 composite response indices: the Paulus criteria, the ACR criteria for improvement in RA, and a reduction of ≥30% in both tender and swollen joint counts. In the 20 μg/day treatment group, this analysis revealed statistically significant improvement in the percentage of patients who met the cumulative Paulus criteria during the study (21 of 54, or 39%) versus the placebo group (11 of 57, or 19%; $P = 0.035$ by Fisher's exact test). This difference remained significant when potential confounding factors (investigative site, baseline use of NSAIDs or steroids, rheumatoid factor positivity, functional class, baseline joint counts, ESR, age, sex, duration of disease, and analgesic use) were accounted for in a logistic regression model ($P = 0.017$ by Wald chi-square test) (25). The percentage of responders (according to the Paulus criteria) in the other 3 treatment groups decreased incrementally with each increase in dosage of CII (Figure 1). No statistically significant interactions between the treatment and any of the covariates were observed.

Response rates for each of the 3 composite

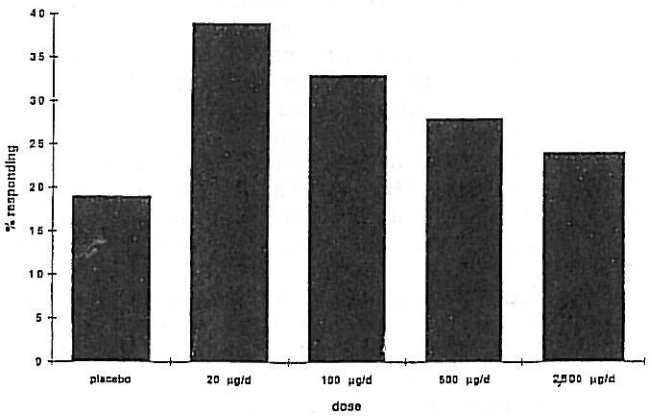

Figure 1. Percentage of patients with rheumatoid arthritis, in the intent-to-treat analysis, meeting the Paulus criteria for response to treatment with type II collagen (CII) versus placebo at any time during the study. The difference in response rates for placebo versus 20 μg/day of CII was statistically significant ($P < 0.05$).

Table 2. Cumulative response rates for composite response criteria, by treatment group*

Composite measure	Placebo	Oral type II collagen 20 μg/day	100 μg/day	500 μg/day	2,500 μg/day
Paulus criteria	19.3	38.9†	32.7	28.3	24.1
ACR criteria	17.5	25.9‡	20.0	24.5	22.2
≥30% criteria§	31.6	46.3¶	40.0	43.4	40.7

* Values are percentages. ACR = American College of Rheumatology.
† $P = 0.035$ vs. placebo.
‡ $P = 0.360$ vs. placebo.
§ ≥30% reduction in both swollen joint count and tender joint count.
¶ $P = 0.123$ vs. placebo.

criteria sets are shown in Table 2. Following the same statistical analysis plan, numeric trends in favor of the 20 μg/day group were seen using the ACR criteria and the ≥30% reduction criteria, although there was no clear difference in response rates between the doses used. Since followup evaluations of functional status were conducted only at weeks 12 and 24, when the HAQ was administered, it is important to note that the ACR criteria were therefore assessed at fewer time points than the other criteria. The inclusion of functional status improvement as a feature of the ACR criteria thus resulted in lower absolute numeric values for responders in this study.

The 4 individual measures selected as primary efficacy end points were swollen and tender joint counts, and physician and patient global assessments of disease activity. Changes in these parameters among the treatment groups during the study are shown in Figures 2A–D. Numeric trends in favor of the 20 μg/day group versus the other treatment groups were seen for 3 of the 4 variables (tender joint count, and physician and patient global scores), although there were no statistically significant differences between the treatment groups at 24 weeks.

There were no statistically or clinically significant changes from baseline, or differences between treatment groups, in any of the measured blood or urine safety

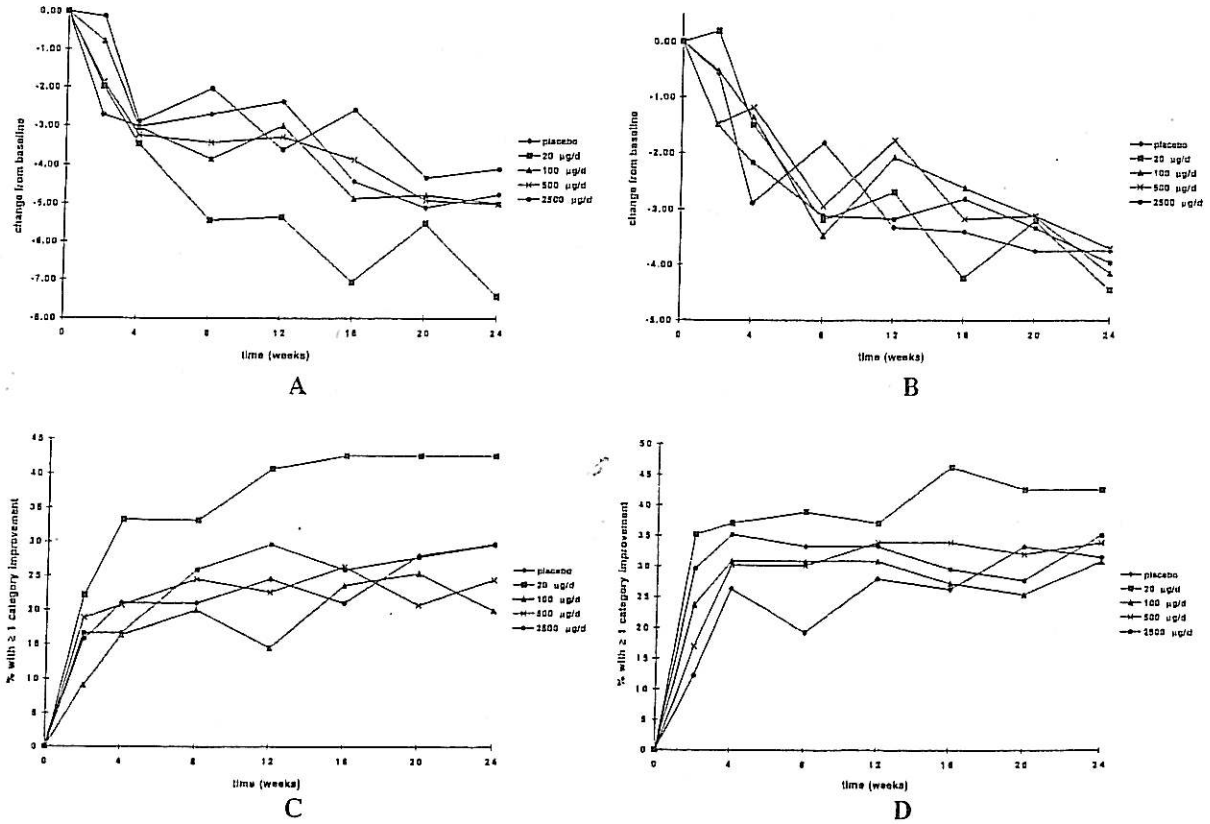

Figure 2. Changes in individual efficacy variables from baseline to 24 weeks by treatment group (intent-to-treat population). A, Tender joint count. B, Swollen joint count. C, Percentage improvement in the physician global score for disease activity. D, Percentage improvement in the patient global score for disease activity.

parameters. A review of individual patient values at baseline revealed hematologic abnormalities typical of patients with RA. Serum and urine chemistry results were unremarkable during the study, except in 6 patients who developed transaminase elevations greater than twice the upper limit of the reference range. These 6 patients included 1 in the placebo group, 1 in the 20 μg/day treatment group, 1 in the 100 μg/day group, and 3 in the 2,500 μg/day group. The highest transaminase level recorded was an alanine aminotransferase value of 583 IU. Three of the 6 patients stopped taking the study medication, resulting in normalization of their transaminase levels, which was followed by a rechallenge with CII and no subsequent liver enzyme abnormalities. The other 3 patients also discontinued the study medication, with subsequent normalization of their transaminase levels; these 3 patients were not rechallenged with CII. Overall, there were no differences between the treatment groups in the incidence of any adverse event during this study (data available on request), and no serious adverse event was attributed to CII.

At baseline, serum IgA and IgG antibodies to CII were detected in 37% and 18% of patients, respectively. When the aggregate group of all patients treated with any dosage of CII was compared with the total placebo group, the presence of anticollagen antibodies was significantly associated with an increased likelihood of achieving a clinical response to CII as defined by the Paulus criteria. Among patients with IgA antibodies present at baseline, 28 (39.4%) of 71 CII-treated patients responded, while only 4 (13.8%) of 29 patients in the placebo group responded. Among patients with IgG antibodies at baseline, 15 (45.5%) of 33 CII-treated patients responded as compared with 2 (13.3%) of 15 patients in the placebo group. These differences were statistically significant ($P < 0.02$ for IgA positivity and $P < 0.05$ for IgG positivity, by Fisher's exact test). There was no significant change in IgA or IgG antibody status with treatment, suggesting that sensitization to CII did not occur. Levels of CRP at baseline did not correlate statistically with the likelihood of response, nor did they change significantly with therapy.

DISCUSSION

We report herein the results of the first multicenter study of oral tolerance in the treatment of human autoimmune disease. Utilizing the cumulative Paulus criteria for response, CII at a dosage of 20 μg/day was shown to be superior to placebo in the treatment of RA. Although there was no clear difference between the dosages used, numeric trends in favor of the 20 μg/day dosage were also seen using the 2 other composite criteria sets, as well as using 3 of the 4 primary individual efficacy parameters that were followed up. As a group, the patients studied in this trial had severe arthritis, with a mean ± SD duration of disease of 11.2 ± 9.1 years. At study entry, patients had a mean ± SD of 24 ± 11 swollen joints and 27 ± 11 tender joints, and 85% of the patients had taken DMARDs previously. While statistically significant improvement was demonstrated with only 1 of the 3 composite criteria sets applied, the mild antirheumatic properties of CII observed in this study must be interpreted in the context of the disease duration and severity in the cohort. Furthermore, CII was shown to have an excellent safety profile that was clinically and immunologically indistinguishable from that of placebo. The high completion rate of 83% of patients enrolled further attests to the tolerability of CII.

A previous study by Trentham et al (10) revealed a positive clinical effect in patients treated with CII at a dosage of 100 μg/day for the first month and 500 μg/day for months 2 and 3. The dose range in the present study was chosen to incorporate these previously studied dosages, in addition to both a lower and a higher dosage, to try to elucidate the most effective level of treatment. The clinical effects seen at the 100 μg/day and 500 μg/day dosages in the present study were not as great as those reported in the previous dose-escalation study (10), but the patients in that trial had less severe disease at baseline and no DMARD washout was required, both of which may have contributed to this observed difference. The recent study of oral type II collagen in the treatment of RA by Sieper et al (11) revealed a higher prevalence of responders among collagen-treated patients versus those receiving placebo, although statistically significant differences between treatment groups were not found. In the study by Sieper et al, bovine, rather than chicken, collagen was used, and the dosages given (1 mg/day and 10 mg/day) were higher than those utilized in the present study. Nonetheless, their finding of a good response in a subset of patients is intriguing, and it leads one to speculate that an immunologic difference might exist that could render some patients more likely to respond to this particular form of therapy.

This study has several limitations. The use of 3 composite criteria sets as end points makes it more difficult to draw conclusions about the clinical efficacy of CII. Given that a statistically significant effect was demonstrated with only 1 of the 3 composite criteria sets tested, it would be premature to interpret these results as conclusive evidence of efficacy. However, since this was a phase II trial, a

primary objective was to gather as much information as possible about effectiveness, toxicity, and optimal dose. Analyzing response rates using a cumulative-response standard can also be criticized, because patients who simply exhibit oscillations in their disease activity (rather than true response to treatment) might be counted as responders. Although this is a valid concern, response rates were compared with those in a placebo group of patients who had an equal likelihood of similar disease fluctuations. Because patients were divided among 5 different treatment groups, the numbers of patients in each group were relatively small, particularly for a multicenter trial. In addition, patients in this study tended to have severe disease of long duration, and therefore, no conclusions can be reached about the use of CII to treat patients with milder or early-stage RA. The dosages of CII tested in this study may not have been optimal, given that the lowest dosage showed the greatest efficacy. Ideally, dosages both lower and higher than the most effective dosage should have been included. Finally, the format of this trial precluded knowing whether the same dosage of collagen is best for all patients.

Our finding that the 20 μg/day dosage proved to be most effective, and that the response rates decreased with each successive increase in dosage, is consistent with observations in animal studies. Active suppression of inflammation occurs in animal models with lower doses of oral tolerogen, but not with higher doses at which anergy and clonal deletion of T cells reactive against the fed antigen are seen (13,14). In a rat model of adjuvant arthritis, the dosage of oral CII most effective in suppressing disease was 3 μg/day (7). The mechanism of oral tolerance is believed to involve the generation of an immune response in the gut, in a manner analogous to oral vaccinations. Given that dosages of oral vaccines are not routinely adjusted based on body weight, the efficacy seen with the lowest dosage tested in the present study is believed to be consistent with the animal data.

Orally dosed exogenous antigen has recently been reported to activate CD4+ and noncytotoxic CD8+ regulatory T cells that may contribute to a down-regulation of cellular and humoral immunity (26,27). The regulatory cells that orchestrate active suppression appear to act via the secretion of inhibitory cytokines, such as TGFβ and IL-4 (12,27). It is presumed that active suppression of inflammation by these regulatory lymphocytes requires migration of these cells to a local microenvironment, which contains a protein resembling the orally dosed heterologous antigen. Because the regulatory cells generated by oral tolerization are primed in an antigen-specific manner but suppress in an antigen-nonspecific manner, they mediate "bystander suppression" when they encounter the orally dosed autoantigen at sites of inflammation (28,29). Thus, it may not be obligatory to identify the target autoantigen for a given disease. What is required is to orally administer a protein that is present at the site of inflammation and is capable of inducing regulatory cells to secrete suppressive cytokines. These findings have important implications for the use of oral tolerance as a therapeutic approach for the treatment of T cell–mediated inflammatory autoimmune diseases in humans, in which the inciting autoantigen is unknown or in which there is autoreactivity to multiple autoantigens in the target tissue. The results in the present study, with maximal efficacy being demonstrated with the lowest dosage of CII tested, are consistent with the results of animal trials (7,8) and with the hypothesis that the mechanism of action of oral tolerance in this setting is active suppression of inflammation rather than clonal anergy or deletion. This finding also suggests that CII may be a useful therapeutic protein even if type II collagen is not of major pathogenetic importance as an autoantigen in established RA or is only one of multiple autoantigens that are involved.

It is of interest that the presence at baseline of serum antibodies to CII, of either the IgA or IgG isotype, was associated with an increased probability of achieving a clinical response after treatment with CII. The basis for this association remains to be explained. Bystander suppression would be expected to benefit patients regardless of whether autoreactivity to CII is involved in the pathogenesis of RA. It is possible that there is a subset of patients with RA in which autoimmunity to CII is of pathogenic importance. It recently has been reported that the presence of antibodies to CII in patients with early RA may be predictive of rapidly progressive disease (30). In patients with antibodies to CII, oral dosing with CII might trigger antigen-specific mechanisms such as anergy or deletion. Alternatively, it is possible that patients with serum antibodies to CII are more likely to generate regulatory cells which would mediate bystander suppression after oral dosing with CII. Further research is needed to confirm this association and to determine whether screening for collagen antibodies would be warranted in deciding which patients to treat with CII.

The phenomenon of oral tolerance represents a novel approach to the treatment of chronic, disabling autoimmune diseases. To date, no serious adverse events have been noted in animal or human studies of

oral tolerance, and the simplicity and apparent safety of this form of treatment make it extremely appealing. Although efficacy of oral CII at 20 μg/day was found to be statistically significant with only 1 of the 3 composite criteria tested, the excellent safety profile and suggestion of efficacy indicate that additional clinical investigations with this protein are warranted, particularly in patients whose disease has not reached the stage and severity encountered in this trial. These studies should also include further analysis of potential immunologic predictors of response to CII.

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REFERENCES

1. Sewell KL, Trentham DE. Pathogenesis of rheumatoid arthritis. Lancet 1993;341:283–6.
2. Mowat A. The regulation of the immune responses to dietary protein antigens. Immunol Today 1987;8:93–8.
3. Higgins PJ, Weiner HL. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. J Immunol 1988;140:440–5.
4. Bitar DM, Whitacre CC. Suppression of experimental autoimmune encephalomyelitis by the oral administration of myelin basic protein. Cell Immunol 1988;112:364–70.
5. Nagler-Anderson C, Bobber LA, Robinson ME, Siskind GW, Thorbecke GJ. Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen. Proc Natl Acad Sci U S A 1986;83:7443–6.
6. Thompson HSG, Staines NA. Gastric administration of type II collagen delays the onset and severity of collagen-induced arthritis in rats. Clin Exp Immunol 1986;64:581–6.
7. Zhang ZY, Lee CS, Lider O, Weiner HL. Suppression of adjuvant arthritis in Lewis rats by oral administration of type II collagen. J Immunol 1990;145:2489–93.
8. Yoshino S, Quattrocchi E, Weiner HL. Suppression of antigen-induced arthritis in Lewis rats by oral administration of type II collagen. Arthritis Rheum 1995;38:1092–6.
9. Weiner HL, Mackin GA, Matsui M, Orav EJ, Khoury SJ, Dawson DM. Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. Science 1993;259:1321–4.
10. Trentham DE, Dynesius-Trentham RA, Orav EJ, Combitchi D, Lorenzo C, Sewell KL, et al. Effects of oral administration of type II collagen on rheumatoid arthritis. Science 1993;261:1727–30.
11. Sieper J, Kary S, Sörensen H, Alten R, Eggens U, Hüge W, et al. Oral type II collagen treatment in early rheumatoid arthritis: a double-blind, placebo-controlled, randomized trial. Arthritis Rheum 1996;39:41–51.
12. Khoury SJ, Hancock WW, Weiner HL. Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor β, interleukin 4, and prostaglandin E expression in the brain. J Exp Med 1992;176:1355–64.
13. Friedman A, Weiner HL. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. Proc Natl Acad Sci U S A 1994;91:6688–92.
14. Chen Y, Inobe J, Marks R, Gonnella P, Kuchroo VK, Weiner HL. Peripheral deletion of antigen-reactive T cells in oral tolerance. Nature 1995;376:177–80.
15. Barnett ML, Combitchi D, Trentham DE. A pilot trial of oral type II collagen in the treatment of juvenile rheumatoid arthritis. Arthritis Rheum 1996;39:623–8.
16. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
17. Steinbrocker O, Traeger CH, Batterman RC. Therapeutic criteria in rheumatoid arthritis. JAMA 1949;140:659–62.
18. Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen: an experimental model of arthritis. J Exp Med 1977;146: 857–68.
19. Fries JF, Spitz PW, Young DY. The dimensions of health outcomes: the health assessment questionnaire, disability and pain scales. J Rheumatol 1982;9:789–93.
20. Weinblatt ME, Coblyn JS, Fox DA, Fraser PA, Holdsworth DE, Glass DN, et al. Efficacy of low-dose methotrexate in rheumatoid arthritis. N Engl J Med 1985;312:818–22.
21. Matteson EL, Yocum DE, St. Clair EW, Achkar AA, Thakor MS, Jacobs MR, et al. Treatment of active refractory rheumatoid arthritis with humanized monoclonal antibody CAMPATH-1H administered by daily subcutaneous injection. Arthritis Rheum 1995;38:1187–93.
22. Van der Lubbe PA, Dijkmans BAC, Markusse HM, Nässander U, Breedveld FC. A randomized, double-blind, placebo-controlled study of CD4 monoclonal antibody therapy in early rheumatoid arthritis. Arthritis Rheum 1995;38:1097–106.
23. Paulus HE, Egger MJ, Ward JR, Williams HJ, and The Cooperative Systematic Studies of Rheumatic Diseases Group. Analysis of improvement in individual rheumatoid arthritis patients treated with disease-modifying antirheumatic drugs, based on the findings in patients treated with placebo. Arthritis Rheum 1990;33:477–84.
24. Felson DT, Anderson JJ, Boers M, Bombardier C, Furst D, Goldsmith C, et al. American College of Rheumatology preliminary definition of improvement in rheumatoid arthritis. Arthritis Rheum 1995;38:727–35.
25. Cox DR. The analysis of binary data. London: Methuen; 1970.
26. Ke Y, Kapp JA. Oral antigen inhibits priming of CD8+ CTL, CD4+ T cells, and antibody responses while activating CD8+ suppressor T cells. J Immunol 1996;156:916–21.
27. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 1994;265:1237–40.
28. Miller A, Lider O, Weiner HL. Antigen-driven bystander suppression after oral administration of antigens. J Exp Med 1991;174:791–8.
29. Al-Sabbagh A, Miller A, Santos LMB, Weiner HL. Antigen-driven tissue-specific suppression following oral tolerance: orally administered myelin basic protein suppresses proteolipid protein-induced experimental autoimmune encephalomyelitis in the SJL mouse. Eur J Immunol 1994;24:2104–9.
30. Cook AD, Rowley MJ, Mackay IR, Gough A, Emery P. Antibodies to type II collagen in early rheumatoid arthritis: correlation with disease progression. Arthritis Rheum 1996;39:1720–7.

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Research Paper

Safety and efficacy of undenatured type II collagen in the treatment of osteoarthritis of the knee: a clinical trial

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Abstract

Previous studies have shown that undenatured type II collagen (UC-II) is effective in the treatment of rheumatoid arthritis, and preliminary human and animal trials have shown it to be effective in treating osteoarthritis (OA). The present clinical trial evaluated the safety and efficacy of UC-II as compared to a combination of glucosamine and chondroitin (G+C) in the treatment of OA of the knee. The results indicate that UC-II treatment was more efficacious resulting in a significant reduction in all assessments from the baseline at 90 days; whereas, this effect was not observed in G+C treatment group. Specifically, although both treatments reduced the Western Ontario McMaster Osteoarthritis Index (WOMAC) score, treatment with UC-II reduced the WOMAC score by 33% as compared to 14% in G+C treated group after 90 days. Similar results were obtained for visual analog scale (VAS) scores. Although both the treatments reduced the VAS score, UC-II treatment decreased VAS score by 40% after 90 days as compared to 15.4% in G+C treated group. The Lequesne's functional index was used to determine the effect of different treatments on pain during daily activities. Treatment with UC-II reduced Lequesne's functional index score by 20% as compared to 6% in G+C treated group at the end of 90-day treatment. Thus, UC-II treated subjects showed significant enhancement in daily activities suggesting an improvement in their quality of life.

Key words: undenatured type II collagen, osteoarthritis, glucosamine, chondroitin, WOMAC, visual analog scale, Lequesne's Functional Index

INTRODUCTION

Arthritis afflicts approximately 43 million Americans or approximately 16.6% of the US population. The two most common types of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA). OA of the knee and hip is a growing health concern and is the most common forms of arthritis (1-3). Pain and disease can range from very mild to very severe (3). Patients with OA have pain that typically worsens with weight bearing, including walking and standing, and improves with rest (4). Other symptoms include morning stiffness and gelling of the involved joint after periods of inactivity. Currently, OA affects

nearly 21 million people in the United States, accounting for 25% of visits to primary care physicians, and half of all Non-Steroidal Anti-Inflammatory Drugs (NSAID) prescriptions. The diverse clinical patterns of OA are observed in approximately 10% of people older than 60 years thus compromising the quality of life of millions of Americans. In addition, OA costs the North American economy approximately $60 billion per year.

Current treatment of OA includes exercise, heat/cold therapy, joint protection, weight loss, physiotherapy/occupational therapy and medications (3-5). The most common medications include acetaminophen and NSAIDs. Although these drugs are effective for reducing pain associated with OA, they do not reverse the disease. In addition, there are considerable side effects associated with the use of these drugs. As a result, OA sufferers have turned to natural nutraceuticals to ease their pain and discomfort. These products are commonly used because they are well tolerated and considered safe. Nutraceuticals are defined as functional foods, natural products, or parts of food that provide medicinal, therapeutic, or health benefits, including the prevention or treatment of disease. Currently, glucosamine and chondroitin are the two most commonly used nutraceuticals in humans as well as in animals to alleviate pain associated with arthritis (6). However, recent randomized controlled trials and meta-analysis of these supplements have shown only small-to-moderate symptomatic efficacy in human OA (7). An emerging novel nutraceutical ingredient known as UC-II has received considerable attention in the treatment of OA. UC-II is a novel undenatured type II collagen derived from chicken sternum cartilage. Previous studies have shown that undenatured type II collagen is effective in the treatment of RA (8-11), and preliminary human (12) and animal (13) trials have shown it to be effective in treating OA. Obese-arthritic dogs given 4 mg or 40 mg daily dose of UC-II for 90 days showed significant declines in overall pain, pain during limb manipulation and lameness after physical exertion (14). Greater improvement was observed with the 40 mg dose. No adverse effects or significant changes in serum chemistry were noted. Following UC-II withdrawal for a period of 30 days, all dogs experienced a relapse of overall pain, exercise-associated lameness and pain upon limb manipulation. Studies have also shown that small doses of orally administered undenatured type II chicken collagen inhibit killer T-cell attack (15). The present clinical trial evaluated the safety and efficacy of UC-II in the treatment of the knee in OA patients.

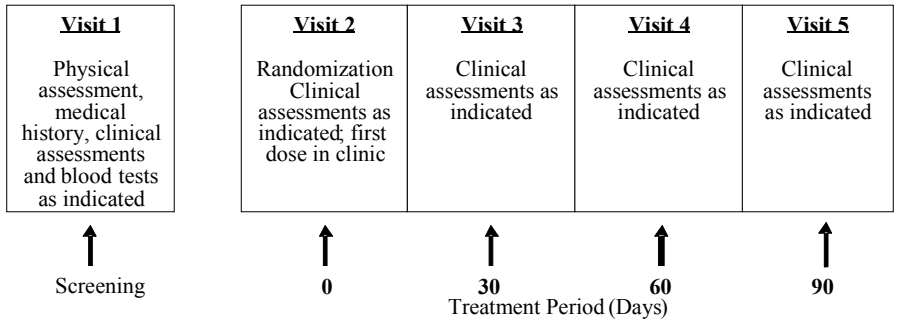


Figure 1. UC-II clinical study design. The study was a two-site, randomized, double-blind study conducted in London, Ontario and Corunna, Ontario, Canada.

Materials and Methods

Study Design

This clinical trial (Human Clinical Trial Approval #06UOHI) was managed by KGK Synergize Inc. (London, ON, Canada). The study was conducted at two sites: 1) KGK Synergize Inc., and 2) Corunna Medical Research (Corunna, ON, Canada). Figure 1 illustrates the study design while Table 1 lists the procedures and observations at each time point.

Briefly, at screening (Visit 1) the consent form was discussed, signed and a complete physical examination was performed. Activity level, diet history, medication/supplement use and medical history were recorded. The VAS score, the WOMAC Index and Lequesne scores were obtained. Urine was collected for a pregnancy test for women of childbearing potential. A blood sample was taken for determination of uric acid, CBC count and differentiation, albumin, total protein, sodium, potassium, chloride, BUN, creatinine, ALT, AST, bilirubin, erythrocyte sedimentation rate (ESR) and rheumatoid factor. Upon review of blood test results, eligible subjects were instructed to get an X-ray of the affected knees to confirm diagnosis. A total of 52 subjects were recruited using the inclusion and exclusion criteria outlined in Table 2. At the first treatment visit (Visit 2), selected subjects were randomly assigned to receive UC-II (n = 26) or glucosamine HCl plus chondroitin sulfate (n = 26, G+C). On each test day (day 0, 30, 60, 90), subjects were required to come to the clinic for clinical assessment. The clinical assessments included WOMAC, Lequesne's functional index and 100-mm VAS pain scores. A subject treatment diary was completed by each patient throughout the study period to determine side effects, medication use, and product compliance.

Table 1. Schedule of observations and procedures

Procedure	Visit 1 Screening	Visit 2 Day 0	Visit 3 Day 30	Visit 4 Day 60	Visit 5 Day 90
Informed consent	X				
Review inclusion/exclusion	X	X	X	X	X
Medical history including activity level and diet history	X				
Physical examination	X				
Biometric measurements: Weight, height*, heart rate and blood pressure.	X	X	X	X	X
Urine pregnancy test	X				
Concomitant medications	X	X	X	X	X
Blood samples: Uric acid, CBC count and differentiation, albumin, total protein, sodium, potassium, chloride, BUN, creatinine, ALT, AST, bilirubin, ESR, rheumatoid factor	X				X
WOMAC, VAS and Lequesne scores	X	X	X	X	X
X-ray	X				
Randomization		X			
Blood sample: ALT, AST, bilirubin, albumin.			X†	X†	
Knee flexion, Time to walk 50m, Swelling in the knee joint, Time for climbing 10 steps		X	X	X	X
Physician's Global Assessment		X	X	X	X
Subject's Global Assessment		X	X	X	X
Investigational Product dispensed		X	X	X	
Subject Treatment Diary dispensed		X	X	X	
Investigational Product returned Compliance calculated			X	X	X
Subject Treatment Diary returned			X	X	X
Adverse Events			X	X	X

* height was only measured at visit 1

† If acetaminophen use was greater than 2 g/day for more than 7 days

Table 2. Inclusion and exclusion criteria

Inclusion Criteria
Males and females 40-75 years old
Females of childbearing potential must agree to use a medically approved form of birth control and have a negative urine pregnancy test result
Unilateral or bilateral OA of the knee for greater than 3 months (American College of Rheumatology criteria) confirmed by radiologist's report, i.e. X-rays showing osteophytes, joint space narrowing or subchondral bone sclerosis (eburnation)
Erythrocyte sedimentation rate (ESR) < 40 mm/hr
Moderate OA as indicated by Lequesne's functional index score of 4.5-7.5 after 7 day withdrawal of usual medications
Able to walk
Availability for duration of study period (3-4 months)
Subject using other therapies for OA, such as exercise, heat/cold therapy, joint protection and physiotherapy/occupational therapy agrees to continue these therapies as normal avoiding changes in frequency or intensity and to record therapies in the study diary
Subject agrees not to start any new therapies for OA during the course of the study
Able to give informed consent
Exclusion Criteria
History of underlying inflammatory arthropathy; septic arthritis; inflammatory joint disease; gout; pseudogout; Paget's disease; joint fracture; acromegaly; fibromyalgia; Wilson's disease; ochronosis; haemochromatosis; heritable arthritic disorder or collagen gene mutations or rheumatoid arthritis
History of asthma, history of diabetes (Type I or Type II)
Hyperuricemia (urate, males > 480 umol/L, females > 450 umol/L)
Expectation of surgery in the next 4 months
Recent injury in the area affected by OA of the knee, i.e. meniscal tear (past 4 months)
Cartilage reconstruction procedure in the target knee
Severe OA as indicated by Lequesne's functional index score of 8 or greater, after 7 day withdrawal of usual medications
Intra-articular corticosteroid injections in the target knee within the last 3 months
Viscous injections in the target knee within the last 6 months
Hypersensitivity to NSAIDs
Abnormal liver or kidney function tests (ALT or AST > 2 times the upper limit of normal; elevated creatinine, males > 125 umol/L, females > 110 umol/L)

Abnormal findings on complete blood count
History of coagulopathies, history of peptic ulceration and upper GI hemorrhage
Uncontrolled hypertension
History of congestive heart failure, history of allergic reaction to chicken and/or eggs
History of allergic reaction to local anesthetic or to any ingredients in the test product including shellfish
Hyperkalemia (potassium > 6.2 mmol/L)
Anticipated problems with product consumption
History of cancer as well as gastrointestinal, renal, hepatic, cardiovascular, hematological, or neurological disorders
High alcohol intake (>2 standard drinks per day)
Pregnant, breastfeeding or planning to become pregnant during the study
History of psychiatric disorder that may impair the ability of subjects to provide written informed consent
Use of other natural health products, including glucosamine and chondroitin, one month prior to study and during the study, other than multivitamin and mineral supplements containing vitamins and minerals as the sole medicinal ingredients
Use of concomitant prohibited medication (narcotics, oral NSAIDs, topical NSAIDs) within four weeks of randomization
Use of acetaminophen or ibuprofen within 7 days of randomization
Subject is unwilling to stop taking pain medication other than the study medication (for arthritis or other types of pain) or is unwilling to stop taking other medications for the treatment of OA
Any other condition that, in the opinion of the investigator, would adversely affect the subject's ability to complete the study or its measures

Supplements

Each UC-II (InterHealth Nutraceuticals, Inc., Benicia, CA) capsule contained 20 mg UC-II standardized to 5 mg of bioactive undenatured type II collagen. Subjects in the UC-II group were instructed to take two "sugar pills" in the morning to protect blinding and two UC-II capsules in the evening accounting for a daily dose of 40 mg UC-II containing 10 mg of bioactive undenatured type II collagen.

Each G+C capsule contains 375 mg of glucosamine HCl (USP Grade) and 300 mg of chondroitin sulfate (USP Grade). The subjects were instructed to take two G+C capsules in the morning and two in the evening for a daily dose of 1500 mg glucosamine and 1200 mg chondroitin.

Removal of Patients from Therapy or Assessment

The criteria for removal of patients from the study included:

Adverse events

For any adverse event, patients were examined and appropriately managed or the patients would be referred to another medical professional for proper evaluation and treatment. If medical problems were attributed to the trial compounds, then the trial drugs were discontinued and the toxicities were reported.

Personal reasons

As stated in the Consent Form, subjects were able to withdraw from the study for any reason at any time.

Clinical judgment of physician

Subjects were withdrawn from the study (without penalty) if, in the opinion of the treating physician, it was not in the patient's best interest to continue. For instance, if during the course of the study a patient became pregnant, she would be withdrawn from the study because it was not known how the study compounds/medications might affect an unborn child.

Protocol violation

Any subject found to have entered this study in violation of the protocol or failed to follow the study protocol were discontinued from the study at the discretion of the Principal Investigator. Subjects were withdrawn for protocol non-compliance if they adhered to the dosing schedule less than 75% of the time.

Method of assigning patients to treatment groups

Patients were assigned to treatment groups (order of treatments) using computer-generated randomization tables. Patients were not stratified or assigned using any other specific method and were not randomized after stratification or blocking procedures.

Selection of doses in the study

The justification for the daily dose of 40 mg UC-II in capsules (providing 10 mg of undenatured collagen II) is based on efficacy demonstrated in earlier studies (8,9).

Blinding

In order to protect blinding, subjects were given bottles containing product labeled with "AM" or "PM" to distinguish the time in which treatment was to be taken. Each bottle contained descriptions of all potential products to ensure blinding was protected. Additionally, each bottle was labeled with a randomization number. In the event that an adverse effect was considered serious and related to the investigational product, the blind would be broken for

that individual subject.

Neither the patient, nor investigator, nor research staff, were aware which test compound the subject was assigned. Interim analysis was performed in order to write a preliminary report and thus preliminary unblinding occurred by an individual unrelated to the study conduct. Personnel related to analysis, statistics, and report writing remained blinded.

Prior and concomitant therapy

Uses of medications such as narcotics, oral NSAIDS, topical NSAIDS within four weeks of randomization and during the study, were not allowed.

Treatment compliance

Compliance was assessed by capsule count at visits 3, 4, and 5 and review of subject diary.

Efficacy and Safety Variables

Efficacy and safety measurements assessed

Adverse events

During the study, subjects recorded adverse effects in their subject diary. At each visit, the subjects were asked if they experienced problems or difficulties. Any adverse events were documented and recorded in the study record and was classified according to the description, duration, severity, frequency, and outcome. The investigator assessed the adverse events and decided causality. Classifications were as per the Coding Symbol Thesaurus of Adverse Reaction Terms (COSTART) U.S. Food and Drug Administration (16).

Blood tests

Blood samples were taken from all subjects during screening (visit 1) and at end of study (visit 5). Blood samples (approximately 15 ml) were taken from subjects at day 30 and day 60 (visits 3 and 4) for the determination of ALT, AST, bilirubin, and albumin if the subjects had been taking acetaminophen greater than 2 g/day for more than 7 days. All blood samples were analyzed by MDS Laboratory Services (London, Ontario, Canada).

Appropriateness of Measurements

The efficacy and safety assessments used in this study were standard for OA and are widely used and recognized as reliable, accurate, and relevant.

WOMAC scores were determined, at screening, and baseline, as well as at days 30, 60 and 90 as described in Bellamy et al (17). Other objectives also performed at days 0, 30, 60 and 90 included determination of Lequesne's functional index, VAS pain scores, knee flexion, time to walk 50 m, time to climb 10 steps, physician's and subject's global assessment. The Lequesne's functional index is described in Lequesne et al. (18).

Statistical Methods

Sample size of 25 subjects per group was based on the subject number used in Braham et al. (1). To compare UC-II with G+C group, a linear contrast was included in the analysis of variance. Data missing subsequent to 30 days were imputed using the last-observation-carried forward technique. Furthermore, comparisons between the UC-II and G+C groups were made at each visit using analysis of variance, using the baseline visit as a covariate. SAS version 9.1 has been used to perform the statistical analysis. Probability values less than 0.05 were considered statistically significant for between-group comparisons.

Results

Baseline Statistics and Compliance of Trial Subjects

Demographic and baseline characteristics of patients are summarized in Table 3. Overall, the patient profiles with respect to age, sex, height, weight, blood pressure, heart beat and target knee were similar between both treatment groups. Table 4 shows treatment compliance of the trial patients. There were no significant interaction terms or between-group differences for compliances. When compliances were compared at each visit, there were no overall between-group differences among the two treatment groups.

Table 3. Demographic and baseline characteristics of the trial subjects

	UC-II (N=26)	G + C (N=26)
Age (years)	58.9 ± 9.79	58.7 ± 10.3
Sex: male/female (%)	13/26 (50%)	17/26 (65%)
Height (cm)	167.7 ± 9.90	167.0 ± 8.73
Weight (kg)	84.3 ± 17.4	86.6 ± 21.0
Systolic Blood Pressure (mm)	128.2 ± 9.36	126.3 ± 12.5
Diastolic Blood Pressure (mm)	81.9 ± 7.43	79.7 ± 8.60
Heart Rate (bpm)	68.2 ± 7.72	67.4 ± 8.47
Target knee		
Left; n (%)	16 (61.5%)	13 (50%)
Right; n (%)	10 (38.5%)	13 (50%)
Where applicable, values are expressed as mean ± SD		

Table 4. Treatment compliance as assessed during specified visits

Visit	Treatment Group	
	UC-II	G + C
AM Capsule Compliance		
Visit 3	[25] 90.5 ± 19.2	[25] 93.6 ± 11.5
Visit 4	[24] 93.2 ± 9.66	[26] 94.5 ± 11.8
Visit 5	[23] 98.5 ± 5.15	[26] 93.3 ± 11.0
PM Capsule Compliance		
Visit 3	[25] 88.1 ± 18.7	[25] 92.5 ± 12.5
Visit 4	[24] 92.8 ± 8.97	[26] 91.6 ± 12.3
Visit 5	[22] 95.3 ± 9.92	[26] 89.7 ± 12.6

There were no significant interaction terms and between-group differences for compliances. When compliances were compared at each visit, there were no overall between-group differences among the five treatment groups. Values are expressed as [n] mean ± SD.

WOMAC Score

The interaction between visit and treatment was significant in UC-II treated group for "pain walking on flat surface" (p=0.034), "difficulty walking on flat surface" (p=0.038) and "performing heavy domestic duties" (p=0.031) as compared to G+C treated group. There was evidence that UC-II treatment has a significant effect for "ascending stairs" (p=0.013) as compared to G+C treatment. Additionally, when groups were compared at each visit, UC-II was significantly better than G+C for "ascending stairs at 30 days and 60 days" (p=0.019 & 0.040 respectively), "at night while in bed" (p=0.015) at 60 days and difficulty walking on flat surface at 90 days (p=0.035). There were no further statistically significant differences for any other individual WOMAC components or summary scores. Treatment with UC-II was most effective and reduced the WOMAC scores by 33% compared to 14% in (G+C)-treated groups after 90 days. Within-group analysis indicated that treatment with UC-II for 90 days significantly ($p<0.05$) improved WOMAC scores at all treatment time points measured. In contrast, subjects received G+C did not show any statistical significant change in WOMAC scores at Day 90 of treatment (Fig. 2).

VAS Score

The interaction between visit and treatment was non-significant for all VAS components and summary scores. However there was evidence that UC-II treatment had a significant effect for "pain during climbing up and down stairs", "night pain" and "resting pain" (p=0.035, 0.030 and 0.024 respectively). When groups were compared at each visit, UC-II was significantly better than G+C for "night pain" (p=0.040) and "resting pain" (p=0.020) at 60 days and "pain during climbing up and down stairs" (p=0.014) and "resting pain" at 90 days (p=0.034). There were no between-group differences for any of the VAS components or summary scores. Although both the treatments reduced the VAS score, UC-II was found to be more effective with a 40% decrease after 90 days of treatment compared to a 15% decrease in G+C treated groups.

Within-group analysis indicated that subjects on UC-II showed a significant reduction in total VAS scores at Day 60 and Day 90 as compared to baseline. However, subjects on G+C showed a significant reduction in total VAS scores at Day 30 and no significant difference was observed at either Day 60 or Day 90 as compared to baseline (Fig. 3).

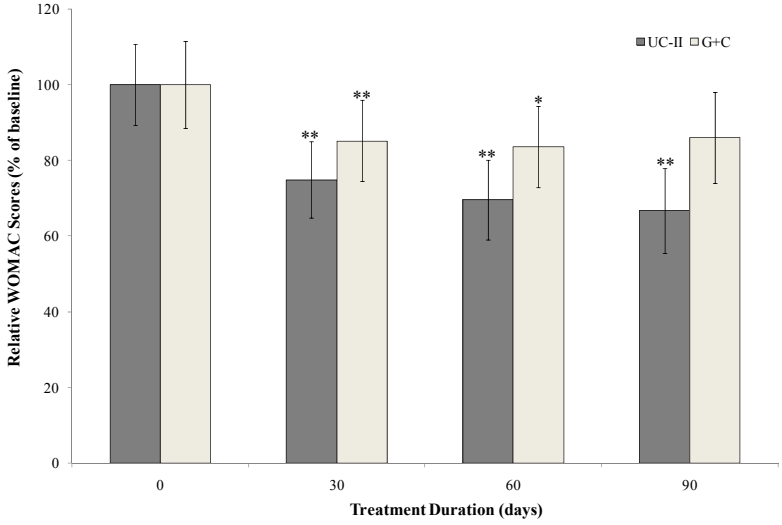


Figure 2. Changes in WOMAC scores at Day 90 from baseline. WOMAC scores from each treatment group were compared to baseline value at specified time points. Each bar presents mean ± SEM. *p<0.05, **p<0.005 indicate significantly different from baseline.

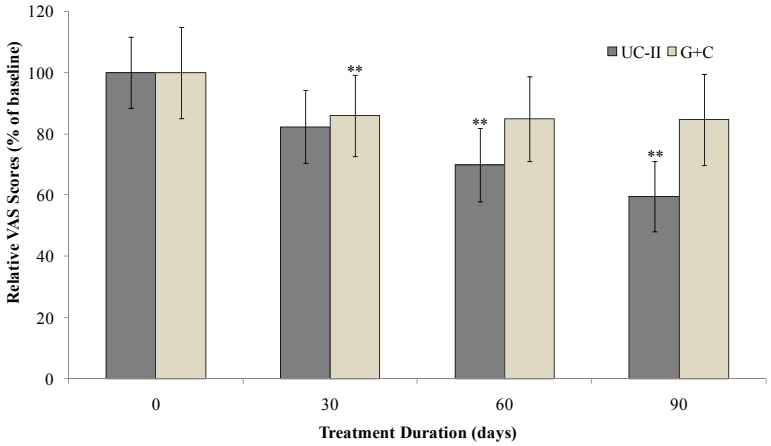


Figure 3. Changes in VAS score at Day 90 from baseline. VAS scores from each treatment group were compared to baseline value at specified time points. Each bar presents mean ± SEM. **p<0.05 indicates significantly different from baseline.

Lequesne Score

The Lequesne's functional index was used to determine the effect of different treatments on pain during daily activities. The interaction between visit and treatment was non-significant for all Lequesne's components and summary scores. Furthermore, there were no between-group differences for any of the Lequesne's components or summary scores. However there was evidence that visit has a significant effect in UC-II treated group for "pain while up from sitting" and "maximum distance walked" (p=0.036 and 0.002 respectively) as compared to G+C treated group.

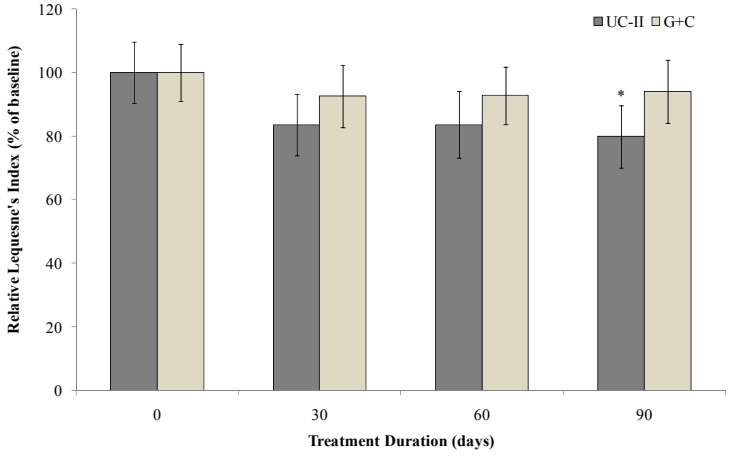
There was as a strong trend toward UC-II efficacy. UC-II treatment effectively reduced Lequesne's functional index score by 20.1% as compared to 5.9 % by G+C treatment.

Within-group analysis suggested that subjects on UC-II demonstrated a significant reduction in total Lequesne's index of severity score from baseline to Day 90, whereas no significant difference from baseline was observed for subjects on G+C at any treatment time points evaluated (Fig. 4).

Figure 4. Changes in Lequesne's functional index at Day 90 from baseline. Lequesne's functional index from each treatment group was compared to baseline value at specified time points. Each bar presents mean ± SEM. *p<0.05 indicates significantly different from baseline.

Adverse Events

Adverse effects that occurred during the 90-day trial period are summarized in Table 5. Overall, there were 58 adverse events noted in the subjects receiving G+C treatment, whereas, only 35 adverse events were observed in UC-II group. In terms of severity, 60% of mild and 38% of moderate adverse events were experienced by subjects on G+C in comparison to 43% and 54% by subjects on UC-II. In relationship to test product a higher number of subjects (23%) on G+C demonstrated adverse events possibly related to product as compared to 11.4% of subjects on UC-II. For UC-II the possible adverse events related to products were constipation and headaches (intermittently). For G+C the possible adverse events related to products were bloating, stomach pain, rash, water retention (edema around eyes and scars), hives on face and chest, and headache. However, there was no significant difference in the occurrence of adverse effects between the two treatment groups.



Rescue Medication

A greater percentage of subjects used rescue medication while on G+C as compared to UC-II at every time point assessed. From baseline to Day 30 a total of 8 subjects (33.3%) on UC-II used rescue medication as compared to 23 subjects (88.5%) on G+C. From Day 30 to Day 60, 13 subjects (54.2%) on UC-II used rescue medication as compared to 21 subjects (80.8%) on G+C. Fourteen subjects (63.6%) on UC-II used rescue medication as compared to 19 subjects (79.2%) on G+C from Day 60 to Day 90.

Table 5. Summary of analysis of adverse events in all subjects

	Treatment Group	
	UC-II (n=26)	**G + C (n=26)**
Severity (n)		
Mild	15	35
Moderate	19	22
Severe	1	1
Relationship to Test Article (n)		
Not related	17	20
Unlikely	14	30
Possible	4	8
Probable	0	0
Most Probable	0	0
Body System (n)		
Pain	10	17
Gastrointestinal	5	15
Musculoskeletal/Soft Tissue	7	5
Neurology	0	2
Pulmonary / Upper Respiratory	2	1
Hemorrhage/Bleeding	2	1
Blood/Bone Marrow	2	1
Dermatology/Skin	2	3
Allergy / Immunology	0	1
Infection	1	3
Lymphatics	0	1
Hepatobilary / Pancreatic	0	0
Renal / Genitoruinary	0	0
Constitutional Symptoms	2	3
Syndromes	1	1
Auditory/Ear	0	1
Ocular / Visual	0	1
Metabolic / Laboratory	1	2
Total Number of Adverse Events Experienced During Study (n)	35	58
Total Number of Subjects Experiencing Adverse Events: n (%)	16/26 (61.5%)	20/26 (76.9%)

Discussion

OA is the most common form of arthritis, and it is often associated with significant disability and an impaired quality of life. Clinical and radiographic surveys have found that the prevalence of OA increases with age from 1% in people <30 years to 10% in those <40 years to more than 50% in individuals >60 years of age (19). Although there are no curative therapies currently available for OA, individualized treatment programs are available to help relieve pain and stiffness, and to maintain and/or improve functional status.

In the last few years, various nutritional supplements including chondroitin, glucosamine, avocado/soybean unsaponifiables and diacerein have emerged as new treatment options for osteoarthritis (20). In this study, the efficacy of UC-II was studied in patients identified with moderate to severe OA. The objective of this study was to determine the effect of UC-II on disease specific measures and blood measures of OA of the knee compared to G+C. It was hypothesized that UC-II would reduce symptoms of OA of the knee to a greater extent than G+C.

A meta-analysis of 20 randomized control studies (2570 patients) comparing the effects of glucosamine (glucosamine sulphate, GS or glucosamine HCl, GH) vs. placebo was done. Of these only eight studies met the required controlled conditions for adequate

allocation concealment and received a quality score of 4 or higher (rated on the JADAD scale). These studies failed to show the benefit of glucosamine (GS or GH) for pain and WOMAC function. When all 20 studies were included in the meta-analysis, the results favored glucosamine with improvement in pain and functionality; however, the results were not uniformly positive and the parameters for WOMAC pain, daily function and stiffness did not reach statistical significance. Combinations of glucosamine and chondroitin have been studied in the "GAIT" study. These authors reported that glucosamine HCl and chondroitin sulphate alone or in combination did not reduce pain significantly in patients with OA of the knee. However in a subgroup of patients with moderate to severe knee pain the combination of compounds were found to be effective. Limitations to this study included a high rate of response to placebo (60.1%) and the fact that 78% of the participants were in the mild pain subgroup (21).

Previous studies have shown that UC-II is effective in the treatment of RA (8-11), and preliminary human (12) and animal (13-15) trials have shown it to be effective in treating OA. In obese-arthritic dogs given 4 mg or 40 mg per day UC-II for 90 days, significant declines in overall pain, pain during limb manipulation and lameness after physical exertion were noted (15). Greater improvement was observed with the 40 mg dose. No adverse effects or significant changes in serum chemistry (creatinine, blood urea nitrogen, alanine aminotransferase, and aspartate aminotransferase) were noted. Following UC-II withdrawal for a period of 30 days, all dogs experienced a relapse of overall pain, exercise-associated lameness and pain upon limb manipulation.

In a recent investigation, efficacy of UC-II was evaluated in arthritic horses (22). In this study, groups of horses were orally administered with a daily dose of placebo, UC-II at 320, 480 or 640 mg, or a combination of glucosamine (5.4 g) and chondroitin (1.8 g) for 150 days. Horses receiving placebo did not show any improvement in arthritic condition, while those receiving a daily dose of 320, 480 or 640 mg of UC-II exhibited significant reduction in arthritic pain. Although G+C treated group showed significant reduction in pain compared to baseline values, the efficacy was less as compared to that observed with UC-II treatment. In fact, UC-II at 480 or 640 mg/day was found to be more effective than G+C in treatment of arthritic pain in horses. Clinical conditions (body weight, body temperature, respiration rate, and pulse rate), and liver (bilirubin, GGT, and ALP) and kidney (BUN and creatinine) functions were not affected by UC-II treatment, suggesting that UC-II is well tolerated and does not cause any adverse effects (22).

In a preliminary trial of subjects with OA, taking a single oral daily dose of 40 mg UC-II on an empty stomach prior to bedtime for 42 consecutive days, an average of 26% reduction of pain was noted in four of five subjects in the study. No side effects were associated with treatment (12). The precise biochemical mechanism involved in UC-II induced pharmacological anti-arthritic effects in humans, dogs or horses is not clearly established. Type II collagen is the primary form of collagen contained in cartilage. Type II collagen extracts contain the amino acids found in the framework of human cartilage. In addition, these amino acids are required for the synthesis and repair of connective tissue throughout the body. These products reportedly aid in reducing the destruction of collagen within the body, may provide anti-inflammatory activity, and may improve joint flexibility (8-12).

The current study indicated that both treatments reduced the WOMAC scores, which measures the difficulty in physical function, stiffness and pain in the knee. However, treatment with UC-II was found to be more effective in reducing the WOMAC scores by 33% as compared to 14% in G+C treated groups after 90 days. Similar results were observed for VAS scores. Although both the treatments reduced the VAS score, UC-II was found to be more effective with 40% decrease after 90 days of treatment as compared to 15.4% in G+C treated groups. The Lequesne's functional index was used to determine the effect of different treatments on pain during daily activities. Treatment with UC-II reduced Lequesne's functional index by 20.1% as compared to 5.9 % in G+C treated groups. Thus, UC-II supplementation showed improvement in daily activities suggesting an improvement in overall quality of life in the patients receiving UC-II.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

References

1. Braham R, Dawson B, Goodman C. The effect of glucosamine supplementation on people experiencing regular knee pain. Br J Sports Med 2003; 37:4549.
2. Cote LG. Management of osteoarthritis. J Am Acad Nurse Pract 2001; 13:495-501.
3. [Internet] Arthritis Foundation. http://www.arthritis.org/conditions/DiseaseCenter/default.asp

4. ANON. Recommendations for the medical management of osteoarthritis of the hip and knee: 2000 update. American College of Rheumatology Subcommittee on Osteoarthritis Guidelines. Arth Rheum 2000; 43:1905-1915.
5. Haq I, Murphy E, Dacre J. Osteoarthritis. Postgrad Med J 2003; 79:377-383.
6. Brown LP. Pet Nutraceuticals; Inter-Cal Nutraceuticals. US: Arthritis Foundation. 2005.
7. Bruyere O, Reginster JY. Glucosamine and chondroitin sulfate as therapeutic agents for knee and hip osteoarthritis. Drugs Aging 2007; 24:573-580.
8. Barnett ML, Kremer JM, St Clair EW, Clegg DO, Furst D, Weisman M, Fletcher MJ, Chasan-Taber S, Finger E, Morales A, Le CH, Trentham DE. Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo-controlled trial. Arthritis Rheum 1998; 41(2):290-7.
9. Barnett ML, Combitchi D, Trentham DE. A pilot trial of oral type II collagen in the treatment of juvenile rheumatoid arthritis. Arthritis Rheum 1996; 39(4):623-8.
10. Trentham DE. Evidence that type II collagen feeding can induce a durable therapeutic response in some patients with rheumatoid arthritis. Ann N Y Acad Sci 1996; 778:306-14.
11. Trentham DE, Dynesius-Trentham RA, Orav EJ, Combitchi D, Lorenzo C, Sewell KL, Hafler DA, Weiner HL. Effects of oral administration of type II collagen on rheumatoid arthritis. Science 1993; 261(5129):1727-30.
12. Bagchi D, Misner B, Bagchi M, Kothari SC, Downs BW, Fafard RD, Preuss HG. Effects of orally administered undenatured type II collagen against arthritic inflammatory diseases: a mechanistic exploration. Int J Clin Pharmacol Res 2002; 22(3-4):101-10.
13. Deparle LA, Gupta RC, Canerdy TD, Goad JT, D'Altilio M, Bagchi M, Bagchi D. Efficacy and safety of glycosylated undenatured type-II collagen (UC-II) in therapy of arthritic dogs. J Vet Pharmacol Ther 2005; 28(4):385-90.
14. D'Altilio M, Peal A, Alvey M, Simms C, Curtsinger A, Gupta RC, Canerdy TD, Goad J, Bagchi M, Bagchi D. Therapeutic efficacy and safety of undenatured type II collagen singly or in combination with glucosamine and chondroitin in arthritic dogs. Tox Mech Methods 2007; 17:189-196.
15. Faria AM, Weiner HL. Oral tolerance. Immunol Rev 2005; 206:232-59.
16. COSTART: Coding symbol thesaurus for adverse reaction terms. 3rd ed. Rockville, MD: Center for Drugs and Biologics, Division of Drug and Biological Products Experience, 1989.
17. Bellamy N, Watson Buchanan W, Goldsmith CH, Campbell J, Stitt LW. 1988. Validation study for WOMAC: health status instrument for measuring clinically important patient relevant outcomes to antirheumatic drug therapy in patients with osteoarthritis of the hip or knee. J Rheumatol 1988; 15:1833-1840.
18. Lequesne MG, Mery C, Samson M, Gerard P: Indexes of severity for osteoarthritis of the hip and knee validation-value in comparison with other assessment tests. Scand J Rheumatol 1987; 65:85-9.
19. Felson DT. The epidemiology of knee osteoarthritis: Results from the Framingham Osteoarthritis Study. Semin Arthritis Rheum 1990; 20:42-50.
20. Van Sasse JL, van Romunde LK, Cats A, Vanderbroucke JP. Epidemiology of osteoarthritis: Zoetermeer survey. Comparison of radiological osteoarthritis in a Dutch population with that in 10 other populations. Ann Rheum Dis 1989; 48(4):271-80.
21. Clegg DO, Reda DJ, Harris CL, et al. Glucosamine, Chondroitin Sulfate, and the Two on Combination for Painful Knee Osteoarthritis. N Engl J Med 2006; 354(8): 795-808.
22. Gupta RC, Bagchi D, Skaggs P, Burke R, Wegford K, Goad JT, Canerdy TD, Barnett D and Bagchi M. Therapeutic efficacy of undenatured type-II collagen (UC-II) in comparison to glucosamine and chondroitin in arthritic horses. J Vet Pharmacol Ther 2008; in press.

ORAL TYPE II COLLAGEN TREATMENT IN EARLY RHEUMATOID ARTHRITIS

A Double-Blind, Placebo-Controlled, Randomized Trial

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Objective. To investigate the efficacy of oral type II collagen in the treatment of early rheumatoid arthritis (RA).

Methods. Ninety patients with RA (disease duration ≤3 years) were treated for 12 weeks with oral bovine type II collagen at 1 mg/day (n = 30) or 10 mg/day (n = 30) or with placebo (n = 30), in a double-blind randomized study.

Results. There was no significant difference between the 3 groups in terms of response to treatment. However, we observed a higher prevalence of responders in the type II collagen–treated groups: 7 responders in the 10-mg type II collagen group and 6 in the 1-mg group, versus 4 in the placebo group. Furthermore, 3 patients in the 10-mg type II collagen group and 1 patient in the 1-mg type II collagen group, but no patients in the placebo group, had very good response. A total of 14 patients had to be withdrawn from the study: 2 because of side effects (nausea) and 12 because of lack of efficacy.

Conclusion. Only a minority of patients responded to treatment with oral type II collagen. These results justify further efforts to identify which patients will have a good response to such therapy.

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Oral tolerance therapy has long been recognized as being able to induce peripheral immune tolerance to specific antigen (1,2). Low doses of orally administered antigen favor active suppression of T cell–mediated immune responses, whereas high doses can induce peripheral tolerance. Treatment with orally fed antigens has proved highly effective in various animal models of human autoimmune diseases, including collagen-induced arthritis (3) and adjuvant arthritis (4), two models that resemble human rheumatoid arthritis (RA). Type II collagen has been selected for use in oral tolerance trials in RA because of its restricted location in cartilage (and the eye) and its abundance (5,6). There is no convincing evidence that collagen itself drives the disease (7,8).

Antigen-specific bystander suppression has been described in several animal models as a mechanism of oral tolerance, where the antigen used is not responsible for the chronic immune response in the target organ (4,9). Thus, in the case of RA, T cells primed to type II collagen in the gut would release suppressive cytokines after a second stimulation by type II collagen in the inflamed joint. From animal experiments, there is evidence that the peripheral suppression is mediated by Th2 cytokines such as interleukin-4 (IL-4), IL-10, and transforming growth factor β (TGFβ), secreted by T cells that are specifically activated in the Peyer's patches of the gut (10). Thus, oral tolerance can be regarded as one approach to rectifying imbalance in T cell cytokine levels.

Although there has been debate in the past

about the role of T cells in the pathogenesis of RA (11,12), there is now increasing evidence of their importance. Several investigators have been able to detect T cell cytokines in RA synovial membrane by molecular genetic (13) or immunohistologic methods (14,15). Furthermore, there is evidence that the Th1 cytokine interferon-γ (IFNγ) predominates in RA synovial membrane (13), as it does in animal models of other T cell–mediated autoimmune diseases such as multiple sclerosis (16) and diabetes mellitus (17). Thus, the concept has arisen that Th1 cells secreting mainly IFNγ are responsible for the chronic immune response against an unknown self antigen, while Th2 cytokines such as IL-4 and IL-10 would inhibit such a immune response (18). This provides scope for intervention strategies which aim at a shift from a Th1 to a Th2 pattern. In experimental allergic encephalomyelitis (the animal model of multiple sclerosis), in non-obese diabetic mice (the animal model of diabetes mellitus), and in collagen-induced arthritis (an animal model of RA), a suppressive effect of Th2 clones (10), IL-4 (19), and the IL-4 gene (20) has been demonstrated. In this context, oral tolerance would work via local production ("bystander suppression") of Th2-type cytokines, which are believed to down-regulate the damaging effect of arthritogenic Th1 cytokines.

Recently, the first clinical trial of oral type II collagen in the treatment of RA demonstrated a trend toward improvement in the type II collagen–treated group compared with the placebo group (21). The design of the present study had three important differences from this earlier study: 1) only patients with early RA (disease duration ≤3 years) were included; 2) 2 different doses of type II collagen (1 mg and 10 mg) were compared with placebo; and 3) instead of chicken type II collagen we used bovine type II collagen, which has a higher homology to human type II collagen. In this study we found a slightly higher response rate among type II collagen–treated patients compared with those who received placebo, especially in the 10 mg group, although this difference was not significant.

PATIENTS AND METHODS

Patient recruitment and characteristics. Patients were recruited at 5 rheumatology clinics in Berlin. Inclusion criteria were as follows: 1) a diagnosis of RA according to the 1987 criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) (22); 2) disease duration ≤3 years; 3) no treatment with disease-modifying antirheumatic drugs (DMARDs) in the previous 2 weeks; 4) clinically active RA with at least 4 swollen and tender joints; 5) treatment with a second-line drug according to need assessed by the physician; 6) prednisolone dosage ≤7.5 mg/day during the trial and for the 14 days before the trial, and no intraarticular injections of corticosteroids during the trial; 7) disease onset between the ages of 16 and 65; 8) patient's written consent.

Patients were excluded from the study if they had myocardial insufficiency, renal insufficiency (serum creatinine >2.0 mg/dl), disturbance of liver function (alkaline phosphatase >300 units/liter, serum glutamic oxaloacetic transaminase [SGOT] >50 units/liter, or bilirubin >1.5 mg/dl), malignancy, or a considerably reduced general state of health as determined by the physician.

Design and duration of the study. The study was a controlled, randomized, double-blind, phase II trial that included 3 groups of patients: 2 different dosage regimens of oral bovine type II collagen (1 mg and 10 mg) were tested against placebo. The planned study size was 30 patients per study arm. Study duration was 12 weeks. The study protocol was approved by the ethical committee of the Klinikum Benjamin Franklin of the Freie Universität Berlin.

Concomitant medication. All patients were treated with nonsteroidal antiinflammatory drugs (NSAIDs) throughout the trial. Any change in dosage or preparation was recorded.

Clinical and laboratory assessments. All patients were examined at 0, 4, 8, and 12 weeks after the start of treatment. The following clinical disease variables were assessed (23,24): number of painful joints (28-joint count), number of swollen joints (28-joint count), patient's global assessment of pain on a 10-point numerical rating scale, the Funktionsfragebogen Hannover (FFbH) questionnaire for measuring functional disability (25), patient's global assessment of disease activity on a 10-point rating scale, physician's global assessment of change in disease activity at the end of the treatment (on a 5-point rating scale), and erythrocyte sedimentation rate (ESR). We decided to use the FFbH questionnaire for measuring physical function because it is the best-validated instrument for RA patients living in Germany. The applied version consists of 12 questions concerning activities of daily living. Patients answer on a 3-point scale: 1 = yes, 2 = yes but with difficulty, 3 = no or only with help. Based on these responses, a score of 0 (no function) to 100% (unimpaired function) is generated. The Ritchie articular index (RAI) (26), duration of morning stiffness, grip strength (mean value of 3 measurements with a Martin vigorimeter), rheumatoid factor (RF), and C-reactive protein (CRP) levels were also documented. Radiographs obtained at study entry were reviewed retrospectively by one examiner.

RF was determined by quantitative nephelometric measurement of latex agglutination (N Latex RF; Behring AG, Marburg, Germany). A level >40 units was regarded as positive. CRP was determined by quantitative nephelometric measurement (N Latex CRP reagents; Behring AG). A value >6 mg/ml was considered positive. HLA class II typing was performed by sequence-specific primed polymerase chain reaction (PCR) (27) and consecutive sequencing-based typing after group-specific PCR amplification (28).

To monitor for possible side effects, the following variables were investigated at each visit: subjective condition, clinical status (measurement of weight and temperature, auscultation of heart and lung, abdominal palpation for tenderness and/or resistance, arterial blood pressure mea-

Table 1. Baseline characteristics of the 90 rheumatoid arthritis patients, by treatment group

Characteristic*	Placebo group (n = 30)	1-mg type II collagen group (n =30)	10-mg type II collagen group (n = 30)
Age, mean ± SD years	53 ± 13	52 ± 8	49 ± 11
No. female/no. male	25/5	20/10	22/8
Disease duration, mean ± SD months	10.3 ± 7.8	20.2 ± 12.0	12.3 ± 12.9
Functional status, no.†			
Class I	3	2	3
Class II	13	18	18
Class III	12	10	9
Class IV	1	0	0
Elevation of ESR, yes/no	15/15	22/8	13/17
Elevation of CRP, yes/no‡	16/14	20/10	13/17
RF positive/negative‡	14/16	17/13	16/14
RA-associated HLA subtype, yes/no‡	16/14	19/11	16/14
Prednisolone treatment, yes/no§	8/22	7/23	12/18
Previous DMARD treatment, no.			
None	28	20	22
Gold salts	–	2	–
Auranofin	–	1	–
Methotrexate	–	2	4
Hydroxychloroquine	–	–	1
Sulfasalazine	2	5	3

* ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; RA = rheumatoid arthritis; DMARD = disease-modifying antirheumatic drug.
† According to the revised criteria of the American College of Rheumatology (ref. 53).
‡ As defined in Patients and Methods.
§ Up to 7.5 mg.

surement, and examination of the skin), complete blood cell count including thrombocytes, levels of SGOT, serum glutamic pyruvic transaminase, alkaline phosphatase, gamma glutamyl transferase, and creatinine, and urinalysis.

Criteria for response. According to the definition proposed by the ACR (29), improvement in RA in clinical trials requires a ≥20% improvement in both tender joint count and swollen joint count and an additional ≥20% improvement in at least 3 of the following 5 parameters: patient global assessment of disease activity, patient pain assessment, patient self-assessed disability, physician global assessment of disease activity, and acute-phase reactant (ESR or CRP). For the FFbH, a change of ≥11% has been found to be significant by test–retest comparison (25), so this cutoff was used to designate a ≥20% improvement, and a change of ≥22% to designate a ≥40% improvement. The percentage change for the variables tender and swollen joints, ESR, and FFbH refers to the difference between the value at the end of study and the value at entry. Patient assessments of disease activity and of pain were measured on a 10-point scale. A difference of 1 point corresponded to 10%. A modification was made with regard to the physician's assessment of disease activity. At the end of the study the physician assessed the change in disease activity on a 5-point-scale (1 = much improved, 2 = improved, 3 = unchanged, 4 = deteriorated, 5 = much deteriorated). The value of 2 (improved) corresponded to an improvement of 20%, and the value 1 (much improved) to an improvement of 40%.

Preparation and handling of bovine type II collagen. Isolation of bovine type II collagen from the nasal septum of cows was carried out according to described methods (30,31). The isolated type II collagen was lyophilized and stored at −20°C. For the trial, type II collagen was dissolved in 0.5*M* acetic acid to final concentrations of 0.2 mg type II collagen/ml 0.5*M* acetic acid or 2 mg type II collagen/ml 0.5*M* acetic acid. Vials containing either 5 ml of 0.2 mg type II collagen/ml 0.5*M* acetic acid (1 mg type II collagen group), 5 ml of 2 mg type II collagen/ml 0.5*M* acetic acid (10 mg type

Table 2. Side effects, by treatment group

	Placebo group (n = 30)	1-mg type II collagen group (n = 30)	10-mg type II collagen group (n = 30)
Pruritus	2	2	3
Exanthema	2	0	3
Gastrointestinal symptoms	4	2	4
Headache	0	0	2

Table 3. Number (%) dropouts, by treatment group

	Placebo group (n = 30)	1-mg type II collagen group (n = 30)	10-mg type II collagen group (n = 30)
All dropouts	6 (20)	4 (13)	4 (13)
Dropouts due to disease worsening	5 (17)	4 (13)	3 (10)
Dropouts due to side effects	1 (3)	0 (0)	1 (3)

Table 4. Number (%) responders and nonresponders, by treatment group

Outcome	Placebo group (n = 30)	1-mg type II collagen group (n = 30)	10-mg type II collagen group (n = 30)
Improvement ≥20%*	4 (13)	6 (20)	7 (23)
Improvement ≥40%*†	0 (0)	1 (3)	3 (10)
Unchanged or worsening	26 (87)	24 (80)	23 (77)

* According to the American College of Rheumatology criteria (ref. 29).
† Includes those whose conditions improved by ≥20%.

II collagen group), or 5 ml 0.5*M* acetic acid (placebo group) were distributed once a month to the patients, who kept them in their home refrigerators at 6°C. Every morning patients swallowed this solution diluted in a glass of water (~100 ml), together with a multivitamin preparation to improve the taste.

Statistical analysis. All patients who were enrolled in the study were included in the evaluation. Before unblinding, the following specifications of the study protocol were determined: the response criteria proposed by the ACR (29) would be used for assessment of efficacy; each patient who fulfilled the criteria would be counted as a responder; and all other patients, including those who did not complete the trial, would be counted as nonresponders. The response rates were compared by Fisher's 1-tailed exact test (significance level 5%). If the response rate was comparable with that of methotrexate or gold, the test would have a power of at least 80% (29).

Additionally, mean changes between baseline and the end of the trial were described for the following efficacy parameters: tender joint count, swollen joint count, RAI, ESR, pain and disease activity assessment by the patients, functional capacity, morning stiffness, and grip strength. Analysis of covariance (ANCOVA) was used to compare these parameters, in order to detect any differences between groups. Individual differences (baseline value minus outcome) were adjusted for their starting point according to the model equation of ANCOVA, and these adjusted differences were then evaluated with two 1-tailed nonparametric tests. The Mann-Whitney test and the multivariate O'Brien test (32) were applied. In all tests, P values less than 0.05 were considered significant.

The statistical analysis included all 90 patients. An analysis of only those who completed the trial might have biased the results. We tested a simple method of replacing missing values (substituting the last valid value) and a more complex one (extrapolation by regression). Since the results were the same, the tables present only the first set of data. No replacement was made in the case of the FFbH because the FFbH was answered only twice, so a missing last value could not be estimated.

RESULTS

Patient characteristics at study entry. Ninety patients were enrolled in the study (30 in each group), of whom 67 (74.4%) were women and 23 (25.6%) we men. The mean age was 51 years (range 19–68 Seventy patients (78%) had not been treated prev ously with any DMARDs. Only 2 patients in th placebo group had received DMARD treatment befor the beginning of the study, in comparison with 1 patients in the 1-mg type II collagen group and patients in the 10-mg type II collagen group. Radic graphs from the time of study entry, available for 6 patients, were examined retrospectively by one ob server. Twenty-three patients exhibited erosions and patients had joint space narrowing as radiologic sign of cartilage destruction. Table 1 summarizes the pa tients' characteristics at the start of the trial. The mean duration of disease, the number of patients with ele vated ESR or CRP, the number of RF-positive pa tients, and the number of patients having an RA-associated HLA class II subtype (DRB1*0101, 0401 0404, 0405, and 0408 [33]) were nonsignificantly higher in the 1-mg type II collagen group compared with the 10-mg type II collagen group and the placebo group. The groups were statistically similar in their demographic and clinical characteristics.

Side effects and withdrawals. Side effects were mild and were equally distributed among the groups (Table 2). Only 2 patients had to be withdrawn from the study because of a side effect (nausea in both cases), and 12 patients were withdrawn due to lack of efficacy (Table 3). Both the 1-mg and the 10-mg collagen treatment groups had fewer withdrawals due to disease worsening (4 patients and 3 patients, respectively) compared with the placebo group (5 patients).

Responders. Eighteen of the 90 patients had improvement according to the ACR criteria. One of the placebo group patients was not treated with prednisolone at study entry, but was treated with 7.5 mg prednisolone daily from week 4 until week 12 during the trial. This patient was not counted as a responder although the improvement criteria as described above were fulfilled.

There was no significant difference in response rates among the 3 groups, although more patients fulfilling the ACR criteria for 20% improvement were found in the 2 type II collagen groups than in the placebo group: 4 patients in the placebo group, 6 patients in the 1-mg type II collagen group, and 7 patients in the 10-mg type II collagen group responded (Table 4). Furthermore, 3 patients in the 10-mg type II collagen group and 1 patient in the 1-mg type II collagen group showed a very good response, either with an improvement of ≥40% by the ACR criteria

(patients 4, 6, and 12; Table 5) or with no swollen or tender joints at the end of treatment (patient 2), but no patient in the placebo group responded in this way. The responder patients are described in more detail in Table 5. No remission as defined by the ACR criteria for complete remission (34) occurred in any of the groups. In most cases the improvement started after 4 weeks, and usually showed a steady improvement over the 12-week treatment period (data not shown). In comparisons of the single variables, there were no significant differences between the groups (Table 6).

DISCUSSION

Statistically significant differences were not found in this study comparing treatment with 10 mg type II collagen/day, 1 mg type II collagen/day, and placebo, in terms of either the ACR criteria for improvement in RA (29) or the mean differences in single variables. However, we found that RA patients treated with type II collagen tended to show benefit. This was especially true with the higher dose of type II collagen (10 mg). In the 10-mg treatment group, 7 patients showed at least a 20% improvement, compared with 6 patients in the 1-mg group and 4 in the placebo group. The rate of withdrawals due to disease deterioration was greatest in the placebo group. It is especially worth mentioning that 4 patients in the type II collagen–treated group, but no patient in the placebo group, showed a very good response: 2 patients in the 10-mg collagen group and 1 in the 1-mg collagen group had improvement of at least 40%, and another patient in the 10-mg group had no tender or swollen joints at the end of the study. This is similar to the finding of 4 remissions in the type II collagen–treated group studied by Trentham et al (21).

Our results appear to be in accordance with those of the previous published study on treatment with oral type II collagen in RA (21), despite a different study design. We did not find a significant difference for any of the single variables, and the differences described in Trentham's study were also unimpressive. The salient feature of both studies seems to be the good response, with remission or near-remission, in a minority of patients. It does not seem to make much difference whether chicken or bovine type II collagen is used or whether early or late RA is treated, although no data on the duration of disease among the patients with remission were given in Trentham's report. Our findings indicate that the higher dosage of type II collagen, i.e., 10 mg/day, may be superior to the lower dosage. However, the differences between groups were too small to allow clear conclusions. It should be noted that the patients in the 1-mg type II collagen group had slightly more severe disease at study entry, as judged from the clinical data (Table 1). In most cases, there was a continuous improvement over the 12 weeks among the responders. This could be taken as evidence that a longer treatment period might be more favorable.

In terms of future studies, it is notable that we found no severe side effects among the collagen-treated patients, and that those that were found did not differ among the groups. Furthermore, the number of dropouts was smaller in the type II collagen groups than in the placebo group, making it unlikely that this treatment worsens the disease, although this is a theoretical possibility since absorption of immunogens in the gut is possible (35–37).

If only a minority of patients respond to treatment, it becomes difficult to demonstrate a significant effect. Our findings pose the question of why only a small number of patients respond, and whether these potential responders could be identified prior to treatment. No differences in RF positivity, HLA class II antigens, or other variables were detected between responders and nonresponders, so it is doubtful that these factors play a major role.

It may be that some patients handle type II collagen in the gut differently. A number of possibilities are worth considering in this context. The amount of oral antigen and the nature of the fragments generated seem to be crucial for the induction of oral tolerance. Digestion of the antigen in the gut is essential for the generation of oral tolerance (37,38), and interference with gastrointestinal proteolysis by neutralizing gastric pH might inhibit the induction of oral tolerance (35). The digestion of such a complex molecule as type II collagen might differ considerably between individuals, and it is not yet clear where and how type II collagen is digested in the gastrointestinal tract. This problem might be circumvented in the future if immunodominant type II collagen peptides can be identified and fed or inhaled (39), instead of administering the whole protein.

Furthermore, it has been suggested that induction of oral tolerance might be dependent on an intact mucosa, since a damaged mucosa allows the passage of immunogenic macromolecules (35,37). This could prevent oral tolerance in RA, since nearly all patients are treated with NSAIDs, which have a known mucosa-damaging effect (40,41). Another possibility is that a change of the bacterial gut flora, for example by drugs (42,43) or diet (44), could also influence the

Table 5. Clinical and laboratory parameters of efficacy and patient characteristics in responders*

Patient no./sex	Tender joints	Swollen joints	ESR	Pain	Disease activity	FFbH	Physician assessment	RF	HLA class II DRB1	Disease duration, months	Radiologic findings	Previous DMARD (washout phase)
10 mg type II collagen group												
Patient 1/F												
Baseline	15	12	48	3	3	–						
Study end	9	8	43	1	0	100.0	2	—	+ 0401	36	Erosions in feet	MTX (25 days)
Difference	6.0	4.0	5.0	2.0	3.0	–						
Difference in %	40.0	33.3	10.4	20.0	30.0	–	20					
Patient 2/F												
Baseline	17	12	15	6	5	91.7						
Study end	0	0	10	2	2	95.8	2	–	†	32	No erosions	MTX (5 months)
Difference	17.0	12.0	5.0	4.0	3.0	4.2						
Difference in %	100.0	100.0	33.3	40.0	30.0	<20	20	–				
Patient 3/F												
Baseline	10	8	24	5	5	75.0						
Study end	8	5	27	2	2	83.3	2	–	0101	7	No erosions	None
Difference	2.0	3.0	−3.0‡	3.0	3.0	8.3						
Difference in %	20.0	37.5	−12.5‡	30.0	30.0	<20	20					
Patient 4/F												
Baseline	17	14	28	6	5	25.0						
Study end	1	2	17	1	2	66.7	1	+	0401	16	No erosions	None
Difference	16.0	12.0	11.0	5.0	3.0	41.7						
Difference in %	94.1	85.7	39.3	50.0	30.0	≥40	40					
Patient 5/F												
Baseline	16	10	10	4	4	75.0						
Study end	5	2	12	1	1	95.8	1	–	†	8	No erosions	None
Difference	11.0	8.0	−2.0‡	3.0	3.0	20.8						
Difference in %	68.8	80.0	−20.0‡	30.0	30.0	≥20	40					
Patient 6/F												
Baseline	20	20	8	6	8	66.7						
Study end	10	0	8	2	2	83.3	1	–	†	3	No erosions	None
Difference	10.0	20.0	0	4.0	6.0	16.7						
Difference in %	50.0	100.0	0	40.0	60.0	≥20	40					
Patient 7/F												
Baseline	15	10	6	1	4	100.0						
Study end	9	6	4	3	2	100.0	2	–	0101	4	No erosions	None
Difference	6.0	4.0	2.0	−2.0‡	2.0	0						
Difference in %	40.0	40.0	33.3	−20.0‡	20.0	<20	20					
1 mg type II collagen group												
Patient 8/F												
Baseline	14	16	83	8	8	50.0						
Study end	5	12	74	4	4	66.7	2	+	0101	11	Erosions in hands	None
Difference	9.0	4.0	9.0	4.0	4.0	16.7						
Difference in %	64.3	25.0	10.8	40.0	40.0	≥20	20					
Patient 9/M												
Baseline	12	10	41	6	7	87.5						
Study end	5	4	7	3	4	91.7	2	+	0401	11	No erosions	AUR (10.5 months)
Difference	7.0	6.0	34.0	3.0	3.0	4.2						
Difference in %	58.3	60.0	82.9	30.0	30.0	<20	20					
Patient 10/F												
Baseline	17	15	25	5	6	50.0						
Study end	0	8	26	2	2	70.8	2	–	0101	12	No erosions	None
Difference	17.0	7.0	−1.0‡	3.0	4.0	20.8						
Difference in %	100.0	46.7	−4.0‡	30.0	40.0	≥20	20					
Patient 11/M												
Baseline	3	9	22	5	7	79.2						
Study end	1	1	17	5	5	91.7	2	–	0401	6	No erosions	SSZ (18 days)
Difference	2.0	8.0	5.0	0	2.0	12.5						
Difference in %	66.7	88.9	22.7	0	20.0	≥20	20					

Table 5. *(Cont'd)*

Patient no./sex	Tender joints	Swollen joints	ESR	Pain	Disease activity	FFbH	Physician assessment	RF	HLA class II DRB1	Disease duration, months	Radiologic findings	Previous DMARD (washout phase)
Patient 12/F												
Baseline	28	16	30	6	4	87.5						
Study end	2	0	10	2	1	100.0	1	–	0101	7	No erosions	None
Difference	26.0	16.0	20.0	4.0	3.0	12.5						
Difference in %	92.9	100.0	66.7	40.0	30.0	≥20	40					
Patient 13/F												
Baseline	21	12	36	5	5	62.5						
Study end	6	2	18	3	2	79.2	1	–	0401	10	No erosions	None
Difference	15.0	10.0	18.0	2.0	3.0	16.7						
Difference in %	71.4	83.3	50.0	20.0	30.0	≥20	40					
Placebo group												
Patient 14/F												
Baseline	13	12	11	3	3	91.7						
Study end	0	6	7	0	0	100	2	–	0401	20	No x-ray available	None
Difference	13.0	6.0	4.0	3.0	3.0	8.3						
Difference in %	100.0	50.0	36.4	30.0	30.0	<20	20					
Patient 15/F												
Baseline	17	10	30	8	4	41.7						
Study end	6	4	23	6	4	50.0	2	+	0401	6	No x-ray available	None
Difference	11.0	6.0	7.0	2.0	0	8.3						
Difference in %	64.7	60.0	23.3	20.0	0	<20	20					
Patient 16/F												
Baseline	6	4	44	5	6	79.2						
Study end	3	3	36	2	3	91.7	2	+	0101, 0401	26	No x-ray available	SSZ (7 months)
Difference	3.0	1.0	8.0	3.0	3.0	12.5						
Difference in %	50.0	25.0	18.2	30.0	30.0	≥20	20					
Patient 17/F												
Baseline	8	12	22	5	5	87.5						
Study end	6	6	6	3	3	95.8	2	–	0401	10	No erosions	None
Difference	2.0	6.0	16.0	2.0	2.0	8.3						
Difference in %	25.0	50.0	72.7	20.0	20.0	<20	20					

* Tender and swollen joint values are from a 28-joint count. Pain and disease activity were rated on a 10-point scale. Physician assessment was done on a 5-point scale. ESR = erythrocyte sedimentation rate (mm/hour); FFbH = Funktionsfragebogen Hannover questionnaire (results expressed as percentage of full functional capacity; difference in % as defined in Patients and Methods); RF = rheumatoid factor; DMARD = disease-modifying antirheumatic drug; MTX = methotrexate; AUR = auranofin; SSZ = sulfasalazine.
† HLA subtype not associated with RA.
‡ Negative difference indicates deterioration.

effect of oral tolerance. It has been shown that orally administered lipopolysaccharide, a major component of bacterial cell walls, enhances the induction of oral tolerance (45), and the gut flora of untreated RA patients is significantly different from that of non-RA controls (46). Finally, treatment of animals with IFNγ abrogates oral tolerance (47). This could be taken as evidence that a strong Th1 response counteracts the assumed Th2 response induced by oral tolerance. Various influences can induce or increase a concomitant Th1 response, e.g., viral and bacterial infections or a hormonal imbalance, with glucocorticoids favoring a Th2 response and dehydroepiandrosterone sulfate and its derivative dehydroepiandrosterone favoring a Th1 response (48).

An additional factor as to why only a minority of patients responded could be our patient selection. We chose to treat only RA patients with early arthritis (duration ≤3 years). Since RA normally has a chronic course with eventual destruction of the joint, a treatment that is started early in the disease has a better chance of cure and/or of preventing irreversible joint damage. Furthermore, animal experiments indicate that disease is best prevented if antigen feeding starts before induction of disease or early in disease. However, the main disadvantage of concentrating on early

Table 6. Disease variables in the 2 type II collagen groups and the placebo group*

Variable, group	Mean ± SD value at entry	Difference from entry at week†			Adjusted difference‡
		4	8	12	
ACR core set variables					
Tender joints (28-joint count)					
Placebo	13.8 ± 6.1	2.2 ± 4.9	3.1 ± 5.2	2.7 ± 7.6	3.4 ± 6.7
1 mg collagen	15.7 ± 7.5	3.4 ± 5.7	5.8 ± 6.7	5.3 ± 7.2	5.1 ± 6.4
10 mg collagen	16.3 ± 5.9	2.3 ± 4.8	4.2 ± 7.3	3.5 ± 7.4	3.0 ± 7.1
Swollen joints (28-joint count)					
Placebo	11.3 ± 5.0	1.4 ± 4.0	1.6 ± 3.9	0.8 ± 4.7	1.0 ± 5.1
1 mg collagen	12.6 ± 5.0	1.7 ± 4.4	3.2 ± 5.1	3.3 ± 5.2	3.2 ± 4.9
10 mg collagen	12.5 ± 5.0	2.4 ± 4.7	2.1 ± 6.6	2.6 ± 7.5	2.5 ± 7.0
ESR (mm/hour)					
Placebo	31.9 ± 29.4	0.9 ± 15.7	2.5 ± 11.5	3.3 ± 12.6	3.5 ± 13.5
1 mg collagen	40.9 ± 31.2	7.6 ± 18.3	12.0 ± 24.2	10.0 ± 24.0	8.3 ± 20.1
10 mg collagen	26.5 ± 21.5	−4.2 ± 14.5	−7.8 ± 16.8	−2.5 ± 13.8	−1.2 ± 15.5
Patient assessment of disease activity (10-point rating scale)					
Placebo	5.2 ± 1.8	1.3 ± 2.3	1.1 ± 1.9	1.0 ± 2.0	0.9 ± 1.9
1 mg collagen	4.5 ± 2.4	0.5 ± 2.1	0.1 ± 2.7	0.1 ± 2.7	0.1 ± 2.3
10 mg collagen	4.8 ± 1.9	0.1 ± 1.9	−0.2 ± 2.6	0.0 ± 2.6	0.0 ± 2.5
Patient pain assessment (10-point rating scale)					
Placebo	5.4 ± 2.1	0.8 ± 2.4	1.0 ± 2.1	1.0 ± 1.9	0.6 ± 2.2
1 mg collagen	4.5 ± 2.1	−0.3 ± 2.8	0.0 ± 2.6	0.4 ± 2.0	0.0 ± 2.4
10 mg collagen	5.0 ± 1.9	−0.3 ± 2.6	−0.3 ± 2.6	−0.1 ± 1.5	−0.2 ± 2.5
Patient self-assessed disability (FFbH)§					
Placebo	71.8 ± 18.2			5.7 ± 12.6	
1 mg collagen	76.7 ± 16.9			5.0 ± 13.1	
10 mg collagen	76.0 ± 16.8			1.4 ± 14.8	
Physician assessment of change in disease activity (5-point scale)¶					
Additional variables					
Ritchie articular index					
Placebo	15.4 ± 6.8	2.8 ± 6.6	3.9 ± 9.3	2.6 ± 9.8	3.7 ± 9.9
1 mg collagen	18.6 ± 8.3	4.9 ± 7.4	6.7 ± 10.3	5.6 ± 9.6	5.3 ± 8.1
10 mg collagen	19.4 ± 10.1	3.4 ± 6.4	4.7 ± 11.0	5.2 ± 11.0	4.5 ± 10.1
Morning stiffness (minutes)					
Placebo	117.2 ± 62.3	49.3 ± 53.1	35.0 ± 106.2	48.5 ± 66.6	41.0 ± 57.0
1 mg collagen	82.2 ± 82.3	3.4 ± 160.2	24.3 ± 89.2	26.2 ± 87.3	31.6 ± 73.4
10 mg collagen	98.2 ± 68.6	30.2 ± 54.7	23.9 ± 88.2	49.0 ± 64.3	48.5 ± 49.5
Grip strength (kPa), right hand					
Placebo	36.7 ± 20.7	8.4 ± 16.0	11.9 ± 17.3	8.7 ± 17.6	6.6 ± 16.5
1 mg collagen	51.4 ± 25.6	2.0 ± 13.4	0.5 ± 17.8	0.7 ± 17.9	2.1 ± 17.1
10 mg collagen	50.3 ± 26.5	−4.6 ± 11.0	−0.9 ± 17.4	1.9 ± 20.7	3.0 ± 19.9
Grip strength (kPa), left hand					
Placebo	23.7 ± 16.4	21.8 ± 16.1	22.8 ± 18.0	20.3 ± 16.3	20.1 ± 16.3
1 mg collagen	37.2 ± 22.1	16.8 ± 12.6	16.2 ± 17.3	15.0 ± 17.8	15.2 ± 17.7
10 mg collagen	33.3 ± 19.3	13.8 ± 14.3	16.0 ± 16.0	17.1 ± 18.2	17.2 ± 18.3

* All patients who enrolled (including dropouts) were included in the analysis; n = 30 in each group. ACR = American College of Rheumatology; see Table 5 for other definitions.
† Negative difference indicates deterioration.
‡ Baseline minus week 12, as defined in Patients and Methods.
§ Applied only at study entry and end. Results expressed as percentage of full functional capacity; dropouts not included.
¶ At study end. In the placebo group, 1 patient's condition was judged to be much improved, 17 improved, 5 unchanged, 5 deteriorated, and 2 much deteriorated. In the 1 mg collagen group, 3 patients' conditions were judged to be much improved, 11 improved, 9 unchanged, 4 deteriorated, and 3 much deteriorated. In the 10 mg collagen group, 3 patients' conditions were judged to be much improved, 12 improved, 7 unchanged, 5 deteriorated, and 3 much deteriorated.

RA in a study like this lies in the possibility that non-RA patients may be inadvertently included. Although all patients in the study met at least 4 of the ACR criteria for RA, in the absence of erosions patients with other, RA-like, diseases are likely to be included, despite the reported 85% specificity of the criteria in early RA (22). Diseases that can resemble early RA, such as primary Sjögren's syndrome, do not

normally lead to erosions of cartilage/bone. However, the concept of oral tolerance in a disease like RA relies heavily on the effect of bystander suppression induced by type II collagen in the joint.

Normally, type II collagen is sequestrated at a privileged site, where it is not recognized by the immune system. Only in a cartilage-destroying disease like RA, with formation of new blood vessels and invasion of T cells and macrophages, can it be assumed that T cells migrating from the gut recognize and are stimulated by collagen in the joint. This would mean that a condition that is confined to inflammation in the synovium, without cartilage damage, would not benefit from treatment with oral type II collagen. Individuals with such conditions would probably be included in any group of patients believed to have early RA. In our study, erosions were not a feature that differentiated responders from nonresponders, but radiologic criteria may be less informative early in disease.

An important means of identifying which RA patients are likely to respond to oral/nasal treatment with type II collagen could be their own level of T cell reactivity to the protein prior to the start of treatment. For this purpose it would help to identify one or more immunodominant peptides, because such peptides have proved useful in assessing T cell reactivity in other diseases (49,50). By way of comparison, mice in which arthritis is induced by foreign type II collagen are highly variable in the extent to which they develop reactivity to their own collagen (51). It would also be helpful if the successful induction of oral tolerance could be measured in patients or controls by the release of cytokines of peripheral blood lymphocytes after in vitro stimulation with type II collagen. Forty-eight to 52 hours after stimulation with myelin basic protein, TGFβ-secreting regulatory cells can be detected in Peyer's patches (52).

In conclusion, we found a tendency toward a higher response rate in the type II collagen–treated patients with RA, especially in the 10-mg treatment group, which is encouraging, and, in our opinion, justifies further investigations of oral tolerance in the treatment of this disease. Oral tolerance is an extremely interesting approach toward immunosuppression because it combines nontoxicity with antigen selectivity. Above all, we need to know how to identify which RA patients are likely to respond to oral tolerance therapy.

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REFERENCES

1. Sieper J, Mitchison AN: Therapy with oral collagen type II as new possibility for selective immunosuppression in the therapy of rheumatoid arthritis. Z Rheumatol 53:53–58, 1994
2. Weiner HL, Friedman A, Miller A, Khoury SJ, Al-Sabbagh A, Santos L, Sayegh M, Nussenblatt RB, Trentham DE, Hafler DA: Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. Annu Rev Immunul 12:809–837, 1994
3. Nagler-Anderson C, Bober LA, Robinson ME, Siskind GW, Thorbecke GJ: Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen. Proc Natl Acad Sci U S A 83:7443–7446, 1986
4. Zhang JZ, Lee CSY, Lider O, Weiner HL: Suppression of adjuvans arthritis in Lewis rats by oral administration of type II collagen. J Immunol 145:2489–2493, 1990
5. Poole AR: Cartilage in health and disease. In, Arthritis and Allied Conditions. Twelfth edition. Edited by DJ McCarty, WJ Koopman. Philadelphia, Lea & Febiger, 1993
6. Prockop DJ, Williams CJ, Vandenberg P: Collagen in normal and diseased connective tissue. In, Arthritis and Allied Conditions. Twelfth edition. Edited by DJ McCarty, WJ Koopman. Philadelphia, Lea & Febiger, 1993
7. Desai BV, Dixit S, Pope RM: Limited proliferative response to type II collagen in rheumatoid arthritis. J Rheumatol 16:1310–1314, 1989
8. Terato K, Shimozuru Y, Katayama K, Takemitsu Y, Yamashita I, Miyatsu M, Fujii K, Sagara M, Kobayashi S, Goto M, Nishioka K, Miyasaka N, Nagai Y: Specificity of antibodies to type II collagen in rheumatoid arthritis. Arthritis Rheum 33: 1493–1500, 1990
9. Miller A, Lider O, Weiner HL: Antigen-driven bystander suppression after oral administration of antigens. J Exp Med 174:791–798, 1991
10. Chen Y, Kuchroo VK, Inobe J-I, Hafler DA, Weiner HL: Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265:1237–1240, 1994
11. Panayi GS, Lanchbury JS, Kingsley GH: The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis (editorial). Arthritis Rheum 35:729–735, 1992
12. Firestein GS, Zvaifler NJ: The role of T cells in rheumatoid arthritis. Ann Rheum Dis 52:765, 1993
13. Simon AK, Seipelt E, Sieper J: Divergent T-cell cytokine patterns in inflammatory arthritis. Proc Natl Acad Sci U S A 91:8562–8566, 1994
14. Lindblad S, Ulfgren A-K, Klareskog L, Andersson U: Detection of cytokine-producing cells in the synovial membrane in rheumatoid arthritis (abstract). Arthritis Rheum 37 (suppl 9): S193, 1994
15. Steiner G, Akrad N, Kunaver M, Gal A, Wessely M, Zenz P, Smolen JS: Production of cytokines by T cells in RA synovial membranes. Clin Rheumatol 14:236, 1995
16. Khoury SJ, Hancock WW, Weiner HL: Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation to inflammatory cytokine and differential upregulation of

transforming growth factor beta, interleukin 4, and prostaglandin expression in the brain. J Exp Med 176:1355–1364, 1992
17. Shehadeh NN, La Rosa F, Lafferty KJ: Altered cytokine activity in adjuvant inhihibiton of autoimmune diabetes. J Autoimmun 6:291–300, 1993
18. Liblau RS, Singer SM, McDevitt HO: Th1 and Th2 CD4+ T cells in the pathogenesis of organ specific autoimmune diseases. Immunol Today 16:34–38, 1995
19. Rapoport MJ, Jaramillo A, Zipris D, Lazarus AH, Serreze DV, Leiter EH, Cyopick P, Danska JS, Delovich TL: Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. J Exp Med 178:97–99, 1993
20. Bessis N, Boissier M-C, Caput D, Fradelizi D, Fournier C: IL-4 or IL-13 transfected xenogenic fibroblasts in the treatment of collagen induced arthritis in mice. Clin Rheumatol 14:261, 1995
21. Trentham DE, Dynesius-Trentham RA, Orav EJ, Combitchi D, Lorenzo C, Sewell KL, Hafler DA, Weiner HL: Effects of oral administration of type II collagen on rheumatoid arthritis. Science 261:1727–1730, 1993
22. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA Jr, Mitchell DM, Neustadt DH, Pinals RS, Schaller JG, Sharp JT, Wilder RL, Hunder GG: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31:315–324, 1988
23. Felson DT, Anderson JJ, Boers M, Bombardier C, Chernoff M, Fried B, Furst D, Goldsmith C, Kieszak S, Lightfoot R, Paulus H, Tugwell P, Weinblatt M, Widmark R, Williams HJ, Wolfe F: The American College of Rheumatology preliminary core set of disease activity measures for rheumatoid arthritis clinical trials. Arthritis Rheum 36:729–740, 1993
24. Prevoo MLL, van't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LBA, van Riel PLC: Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 38:44–48, 1995
25. Raspe H-H, Kindel P, Vesterling K, Kohlmann T: Die Entwicklung der Funktionskapazität und der Schmerzintensität von 81 cP-Patienten unter einer Behandlung mit Azulfidine RA oder Aurothioglucose. Z Rheumatol 46:71–75, 1987
26. Ritchie DM, Boyle JA, McInnes JM, Jasani MK, Dalakos TG, Grieveson P, Buchanan WW: Clinical studies with an articular index for the assessment of joint tenderness in patients with rheumatoid arthritis. Q J Med 37:393–406, 1968
27. Olerup O; Zetterquist H: HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 39:225–35, 1992
28. Blasczyk R, van Lessen A, Schwella N, Huhn D, Salama A: A novel HLA DR 13 allele (DRB1*1314) identified by single-strand conformation polymorphism analysis and confirmed by direct sequencing. Hum Immunol 43:303–312, 1995
29. Felson DT, Anderson JJ, Boers M, Bombardier C, Furst D, Goldsmith C, Katz LM, Lightfoot R Jr, Paulus H, Strand V, Tugwell P, Weinblatt M, Williams HJ, Wolfe F, Kieszak S: American College of Rheumatology preliminary definition of improvement in rheumatoid arthritis. Arthritis Rheum 38:727–735, 1995
30. Ricard-Blum S, Tiollier J, Garrone R, Herbage D: Further biochemical and physiochemical characterization of minor disulfide-bonded (type IX) collagen, extracted from foetal calf cartilage. J Cell Biochem 27:147–158, 1985
31. Boissier M-C, Chiocchia G, Ronziere M-C, Herbage D, Fournier C: Arthritogenicity of minor cartilage collagens (types IX and XI) in mice. Arthritis Rheum 33:1–8, 1990
32. O'Brien PC: Procedures for comparing samples with multiple endpoints. Biometrics 40:1079–1087, 1984
33. Ollier W, Thomson W: Population genetics of rheumatoid arthritis. Rheum Dis Clin North Am 18:741–759, 1992
34. Pinals RS, Masi AT, Larsen RA, and the Subcommittee for Criteria of Remission in Rheumatoid Arthritis of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee: Preliminary criteria for clinical remission in rheumatoid arthritis. Arthritis Rheum 24:1308–1315, 1981
35. Bloch K, Bloch D, Stearns M, Walker WA: Intestinal uptake of macromolecules. VI. Uptake of protein antigen in vivo in normal rats and in rats infected with Nippostrongylus brasiliensis or subjected to mild systemic anaphylaxis. Gastroenterology 77:1039–1044, 1977
36. Curtis CH, Gall DG: Macromolecular transport by rat gastric mucosa. Am J Physiol 257:G1033–G1040, 1992
37. Hanson D, Roy M, Green GM, Miller S: Inhibition of orally-induced immune tolerance im mice by prefeeding an endopeptidase inhibitor. Reg Immunol 5:76–74, 1993
38. Michael JG: The role of digestive enzymes in orally induced immune tolerance. Immunol Invest 18:1049–1054, 1989
39. Metzler B, Wraith DC: Inhibiton of experimental autoimmune encephalomyelitis by inhalation but not oral administration of the encephalogenic peptide: influence of MHC binding affinity. Int Immunol 5:1159–1165, 1993
40. Bjarnason I, Williams P, So A, Zanelli GD, Levi A J, Gumpel JM, Peters TJ, Ansell B: Intestinal permeability and inflammation in rheumatoid arthritis: effects of non-steroidal anti-inflammatory drugs. Lancet 2:1171–1173, 1984
41. Bjarnason I, Zanelli G, Smith T, Prouse P, Williams P, Smethurst P, Delacey G, Gumpel MJ, Levi AJ: Nonsteroidal antiinflammatory drug-induced intestinal inflammation in humans. Gastroenterology 93:480–489, 1987
42. Bradley SM, King B, Troughton PR, Gooi HC, Bird HA, Wright V: Clostridium perfringens and antibody responses to its antigen in arthritis patients on and off non-steroidal anti-inflammatory drugs. Br J Rheumatol 32:940–941, 1993
43. Kanerud L, Scheynius A, Nord CE, Haftström I: Effect of sulphasalazine on gastrointestinal microflora and on mucosal heat shock protein expression in patients with rheumatoid arthritis. Br J Rheumatol 33:1039–1048, 1994
44. Peltonen R, Kjeldsen-Kragh J, Haugen M, Tuominen J, Toivanen P, Forre O, Eerola E: Changes of faecal flora in rheumatoid arthritis during fasting and one-year vegetarian diet. Br J Rheumatol 33:638–643, 1994
45. Khoury SJ, Lider O, Al-Sabbagh A, Weiner HL: Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. III. Synergistic effect of lipopolysaccharide. Cell Immunol 131:302–310, 1990
46. Eerola E, Möttönen T, Hannonen P, Luukkainen R, Kantola I, Vuori K, Tuominen J, Toivanen P: Intestinal flora in early rheumatoid arthritis. Br J Rheumatol 33:1030–1038, 1994
47. Zhang Z, Michael JG: Orally inducible immune responsiveness is abrogated by IFNγ treatment. J Immunol 144:4163–4165, 1990
48. Clerici M, Shearer GM: The Th1-Th2 hypothesis of HIV infection: new insights. Immunol Today 15:575–581, 1994
49. Nishimura M, Kermode AG, Clerici M, Shearer GM, Berzofsky JA, Uchiyama T, Wiktor SZ, Pate E, Maloney B, Manns A, Blattner W, Jacobson S: Demonstration of human T lymphotropic virus type I (HTLV-1)-specific T cell responses from seronegative and polymerase chain reaction negative persons to HTLV-1. J Infect Dis 170:334–338, 1994
50. Rowland-Jones S, Sutton J, Ariyoshi K, Dong T, Gotch F, McAdam S, Whitby D, Sabally S, Gallimore A, Corrah T,

Effects of Oral Administration of Type II Collagen on Rheumatoid Arthritis

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Rheumatoid arthritis is an inflammatory synovial disease thought to involve T cells reacting to an antigen within the joint. Type II collagen is the major protein in articular cartilage and is a potential autoantigen in this disease. Oral tolerization to autoantigens suppresses animal models of T cell–mediated autoimmune disease, including two models of rheumatoid arthritis. In this randomized, double-blind trial involving 60 patients with severe, active rheumatoid arthritis, a decrease in the number of swollen joints and tender joints occurred in subjects fed chicken type II collagen for 3 months but not in those that received placebo. Four patients in the collagen group had complete remission of the disease. No side effects were evident. These data demonstrate clinical efficacy of an oral tolerization approach for rheumatoid arthritis.

Rheumatoid arthritis (RA) is a common chronic illness in which the synovial membrane of multiple joints becomes inflamed, causing damage to cartilage and bone. Although the pathogenetic mechanisms underlying the disease are unknown, rheumatoid arthritis is associated with human lymphocyte antigen (HLA)–DR4 and considered to be an autoimmune disorder in which activated T cells participate (*1*). Type II collagen is a candidate autoantigen for this disease because it is the most abundant structural protein of cartilage, and immunization of animals with the native protein creates arthritis morphologically resembling rheumatoid arthritis (*2*, *3*). Patients with the disease have immune responses to native type II collagen (*4*), but whether collagen reactivity participates in the primary pathogenesis of rheumatoid arthritis or reflects tissue degradation is unknown.

Current treatments are inadequate in that they only partially control established rheumatoid arthritis. They also have side effects that limit use early in the disease process and interfere with prolonged administration (*5*). An ideal therapy would decrease inflammation in the joint by a disease-specific mechanism and would lack toxicity. Oral tolerization, a method of inducing antigen-specific tolerance, suppresses animal models of the autoimmune diseases multiple sclerosis, uveitis, and diabetes (*6*–*11*). In a double-blind pilot trial involving 30 patients with multiple sclerosis, oral administration of bovine myelin antigens decreased the number of T cells that reacted with myelin basic protein (MBP), with no measurable toxicity (*12*). Although favorable trends occurred in the myelin group, clinical efficacy could not be determined because of the small sample size.

Oral administration of native type II collagen ameliorates two animal models of rheumatoid arthritis induced by type II collagen (*13*) or complete Freund's adjuvant (*14*). These experimental findings provided the rationale for a pilot, open-label dose-escalation and safety study in 10 patients with recalcitrant rheumatoid arthritis. Subjects were taken off their immunosuppressive and disease-modifying drugs consisting of methotrexate, 6-mercaptopurine, azathioprine, or auranofin and fed 0.1 mg of solubilized type II collagen daily for 1 month and then switched to 0.5 mg for the next 2 months (*15*). This dose was extrapolated from experiments in the rat adjuvant arthritis model where feeding 3 to 30 μg of collagen attenuated disease (*14*) and the rat experimental autoimmune encephalomyelitis (EAE) model where feeding 500 to 1000 μg of MBP was suppressive (*6*, *10*). Six of the 10 patients experienced a substantial clinical response, defined by a ≥50% improvement in both swollen and tender joint counts with two additional disease measures improving by ≥50% [morning stiffness, 15-m walk time, grip strength, Westergren erythrocyte sedimentation rate (ESR), or physician or patient global assessments] and lasting for at least 2 months after the treatment period (*16*). A complete response, that is, disease remission (*17*) with discontinuation of nonsteroidal anti-inflammatory drug (NSAID), occurred in one patient previously on methotrexate and continued for 26 months. There were no adverse effects. Based on the results of this phase I study, a placebo-controlled, phase II trial was undertaken to determine whether clinical efficacy could be demonstrated.

For this phase II trial, 60 patients with severe, active rheumatoid arthritis and who met eligibility criteria (*18*) gave informed consent (*19*) and were entered into the study. They were withdrawn from immunosuppressive drugs if they had been taking them (*20*) and randomized (*21*) to either a treatment identical to that used in the phase I trial (*15*) or an indistinguishable placebo (*22*) to be taken orally for a consecutive 90-day period. Both patients and investigators, except those responsible for medication (*23*), were masked as to treatment. Assessments were performed by the same investigator (D.E.T.) at the initiation of treatment and at 1, 2, and 3 months, generally at the same time of day (*24*).

At the conclusion of the study, 59 of the 60 patients were considered evaluable (*25*); 28 had received collagen and 31 placebo. On entry, demographic, clinical, and laboratory parameters were similar in both groups (Table 1) (*26*). Relative to entry, there was significant ($P < 0.05$) improve-

Table 1. Patient characteristics at entry. There were no differences between groups ($P > 0.10$) detected by either Fisher's exact test or the Wilcoxon rank-sum test (age and disease duration).

Characteristic	Treatment	
	Collagen ($n = 28$)	Placebo ($n = 31$)
Age (years ± SD)	50.3 ± 11.9	55.1 ± 12.9
Sex (% females)	71	68
Disease duration (years ± SD)	9.8 ± 6.2	10.3 ± 8.1
Rheumatoid factor [%, (number tested)]	74 (27)	82 (28)
HLA-DR 4⁺ [%, (number tested)]	46 (28)	62 (29)
Collagen II antibody (%, titer ≥ 2)	32	13
Prednisone (%, ≤10 mg/day)	25	48
Immunosuppressive* withdrawn (%)	64	58

*Methotrexate, 6-mercaptopurine, azathioprine, hydroxychloroquine, sulfasalazine, auranofin, cyclosporin, cyclophosphamide, or penicillamine. Seven patients were receiving combinations of these drugs (*20*). The remaining patients were not on immunosuppressive drugs at the time of entry because of prior lack of response or toxicity to at least two of the drugs.

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ment in the number of swollen joints, the number of tender or painful joints, joint-swelling and -tenderness indices, and 15-m walk time at months 1, 2, and 3 in the collagen group as compared with placebo patients, except for the number of tender or painful joints at month 2 ($P = 0.06$) (Table 2). Among the collagen patients, the decline in the number of swollen joints, tender joints, and joint-swelling and -tenderness indices were all significant ($P < 0.05$). Four of the collagen patients (14%), as compared with none in the placebo group, had complete resolution of disease (*27*). Table 3 indicates the patients' status by other outcome measures (*16, 21, 28*). Stability or improvement while patients were off immunosuppressives occurred in the collagen group, whereas patients in the placebo group tended to deteriorate. In alternative analyses that reduce the influence of the four placebo patients who withdrew from the trial (*25*), a similar significant ($P \leq 0.05$) improvement from collagen was seen (*29*). A placebo effect resembling that encountered in other RA trials (*30*) was also observed. Four patients (13%) in the placebo group exhibited substantial benefit (*16*) and attained functional class I ranking. This observation reaffirms the critical importance of placebo-controlled evaluations in rheumatoid arthritis. No side effects or significant changes in laboratory values, including rheumatoid factor–and antibodies to type II collagen, were noted. There was no evidence of sensitization to collagen, as measured by antibodies to type II collagen. Attempts to assess T cell responses to type II collagen, including release of transforming growth factor–β (TGF-β), were unsuccessful because c difficulty in demonstrating reactivity tc II collagen in the peripheral blood o patients. None of the baseline feat including the presence of collagen anti ies, HLA haplotype, or sex, were assoc with responsiveness to collagen (*31*).

This controlled trial provides evid that oral administration of small quant of solubilized native heterologous typ collagen is both safe and can improve clinical manifestations of active rheuma arthritis. Baseline values were determi while 64% of the collagen-treated pati were on immunosuppressive drugs (usu methotrexate or 6-mercaptopurine), further improvement occurred with co gen treatment. If longer term efficac established, oral collagen would be a p erable treatment because it is not to

Table 2. Disease variables in collagen-versus placebo-treated patients. Number of patients (collagen/placebo) evaluated at entry = 28/31, 1 month = 27/29, 2 months = 26/26, and 3 months = 28/31; withdrawals were treated as described (*25*); values shown are differences from entry except for patient and physician assessments which are given as percentages. There were no significant differences between groups at entry ($P > 0.05$ for all variables by the Wilcoxon rank-sum test or the χ^2 trend test for patient and physician assessments) (*16*). Comparisons between groups showed significantly more improvement or less worsening in the collagen-treated patients ($P < 0.05$ and $P < 0.01$). Differences between physician assessments in collagen and place patients were not significant but showed trends in favor of collagen a month ($P = 0.066$) and 2 months ($P = 0.06$). Qualitatively similar resu were found when a two-way analysis of variance was used to adjust prednisone use. Significant improvement was also observed amo collagen-treated patients at 1, 2, and 3 months in terms of the numb of swollen joints, the swollen joint index, the number of tender joints, a the tenderness index (Student's t test; all P values are <0.01, except 3 months, for the number of swollen joints, $P = 0.02$, and the swoll joint index, $P = 0.03$).

Variable	Group	Mean value at entry (± SE)	Difference from entry at month		
			1	2	3
Joints swollen (number)	Collagen	11.8 ± 0.9	−2.7 ± 0.5**	−4.1 ± 1.0*	−3.1 ± 1.1
	Placebo	12.0 ± 0.8	2.0 ± 1.4	0.9 ± 1.6	1.3 ± 1.4
Joints tender to pressure or painful on passive motion (number)	Collagen	15.8 ± 1.3	−4.1 ± 1.1*	−6.7 ± 1.5	−5.4 ± 1.8
	Placebo	15.6 ± 0.8	1.1 ± 1.4	−1.1 ± 1.7	−0.1 ± 1.6
Joint-swelling index	Collagen	13.3 ± 1.1	−3.4 ± 0.8**	−4.8 ± 1.2*	−3.1 ± 1.4*
	Placebo	13.2 ± 0.9	2.4 ± 1.8	0.9 ± 1.6	4.3 ± 2.1
Joint-tenderness or pain index	Collagen	17.5 ± 1.3	−5.0 ± 1.2**	−7.6 ± 1.7*	−5.7 ± 2.0*
	Placebo	17.2 ± 1.0	1.6 ± 1.8	−0.5 ± 2.1	3.0 ± 2.4
15-m walk time (s)	Collagen	13.2 ± 0.6	0.0 ± 0.3**	0.25 ± 0.5**	0.5 ± 0.6*
	Placebo	14.9 ± 0.9	1.9 ± 0.6	3.8 ± 1.2	20.8 ± 7.5
Grip strength (mmHg)					
Right	Collagen	105 ± 9	0.1 ± 6.0	6.3 ± 7.8	−0.9 ± 8.5
	Placebo	87 ± 8	−7.3 ± 6.2	−8.3 ± 8.4	−16.4 ± 8.8
Left	Collagen	106 ± 10	0.6 ± 5.6	6.6 ± 7.4*	−0.3 ± 8.8
	Placebo	95 ± 8	−8.9 ± 5.8	−9.3 ± 10.1	−13.8 ± 9.7
Morning stiffness duration (min)	Collagen	155 ± 51	64.8 ± 106	51.2 ± 100	56.4 ± 92
	Placebo	210 ± 55	130 ± 76	168 ± 108	195 ± 100
Patient assessment (%)					
Absent or mild	Collagen	21	41	23*	36*
Moderate	Collagen	54	33	46*	25*
Severe or very severe	Collagen	25	26	31*	39*
Absent or mild	Placebo	16	21	15	19
Moderate	Placebo	35	31	23	10
Severe or very severe	Placebo	48	48	62	71
Physician assessment (%)					
Absent or mild	Collagen	18	41	35	32
Moderate	Collagen	46	33	38	29
Severe or very severe	Collagen	36	26	27	39
Absent or mild	Placebo	6	21	27	19
Moderate	Placebo	42	31	12	13
Severe or very severe	Placebo	52	48	62	68
ESR (mm/hour)	Collagen	39 ± 6	5.1 ± 2.9	4.9 ± 2.8	1.7 ± 3.9
	Placebo	34 ± 5	9.8 ± 5.0	7.8 ± 5.6	3.2 ± 2.8

*$P < 0.05$. **$P < 0.01$.

Although it is possible that the disease could be exacerbated or an allergy to the oral antigen could develop, this was not observed in our study, in animals (*6–11, 14*), in multiple sclerosis patients given oral myelin for as long as 3 years (*12*), or in uveitis patients treated with retinal S-antigen (*32*). All patients in the phase II trial and open-label trial had collagen discontinued after 3 months. Four patients in the pilot study who improved while on collagen experienced a relapse about 3 months after cessation of therapy followed by benefit with reinitiation of collagen. In animals, protective effects of oral tolerance appear to last for 2 to 3 months after termination of antigen feeding (*6*). Recrudescence of disease after discontinuation of oral toleragen has also occurred in multiple sclerosis (*12*) and uveitis (*32*) patients. It therefore appears that additional administration is required to maintain the clinical effects of oral tolerance.

On the basis of studies of oral tolerance in animals, two immunologic mechanisms could explain the clinical response to collagen observed in this study. Feeding type II collagen in RA cases may both anergize $CD4^+$ type II collagen autoreactive cells and generate major histocompatibility complex (MHC) class I– or class II–restricted regulatory cells that sequester within joint tissues and release cytokines that inactivate autoaggressive cells. In animals, feeding large doses of antigen favors T cell anergy, whereas multiple small doses favors the induction of regulatory T cells (*33*). In the EAE model, feeding low doses of MBP activates MBP-specific regulatory cells in gut lymphoid tissue (*10*). These cells are predominantly $CD8^+$ and suppress EAE by trafficking to the central nervous system and releasing anti-inflammatory cytokines, such as TGF-β and interleukin-4, when they encounter MBP presented by MHC molecules in inflamed brain tissue. This process, termed antigen-driven bystander suppression (*10*), implies that an orally administered protein can down-regulate organ-specific autoimmune disease as long as it is a constituent of the target tissue and is capable of inducing regulatory T cells. It is not obligatory for the protein to have the disease-inciting epitopes. Examples of bystander suppression include inhibition of proteolipid protein (PLP)–induced EAE by orally administered MBP (*34*), delay of diabetes in the non-obese diabetic mouse by oral insulin (*11*), and abrogation of adjuvant arthritis by oral collagen (*14*). In all three models, autoimmunity to the toleragen does not appear to initiate disease. Accordingly, our data do not determine whether type II collagen is the primary autoantigen in rheumatoid arthritis.

Although initial clinical efficacy of oral collagen has been shown, questions concerning optimum dosing and long-term control of disease remain. Nonetheless, this study demonstrates the therapeutic efficacy of oral tolerance for a human autoimmune disease and provides the foundation for the development of oral collagen as an easily administered nontoxic treatment for rheumatoid arthritis.

Table 3. Outcome measures in collagen- versus placebo-treated patients. Values are percentages of 28 collagen and 31 placebo patients.

Variable	Entry		Three months	
	Collagen	Placebo	Collagen	Placebo
Worsening status*			7*	35
Analgesic use†			14†	39
Functional class‡				
I	0	0	18	13
II	57	58	39	19
III	43	42	39	58
IV	0	0	4	10

*Represents an increase of 30% or more from the entry value for the joint-swelling index and the joint-tenderness or pain index (*16*). Comparison between groups showed significantly more deterioration in the placebo-treated patients ($P < 0.01$ by the Fisher's exact test). †Narcotic without anti-inflammatory properties, usually acetaminophen with codeine, propoxyphene, or pentazocine, prescribed at any time by the clinical investigator in an attempt to retain flaring patients in the trial. Comparison between groups showed significantly greater numbers of placebo-treated patients requiring narcotics ($P < 0.04$ by the Fisher's exact test). ‡Determined by American Rheumatism Association criteria for functional class (*35*): I, no limitation from arthritis; II, mildly restricted; III, markedly restricted; and IV, incapacity causing total bed or wheelchair existence. Trend for improvement in the collagen group not significant ($P = 0.10$ by the χ^2 trend test).

REFERENCES AND NOTES

1. K. L. Sewell and D. E. Trentham, *Lancet* 341, 283 (1993).
2. D. E. Trentham, A. S. Townes, A. H. Kang, *J. Exp. Med.* 146, 857 (1977).
3. J. S. Courtenay, M. J. Dallman, A. D. Dayan, A. Martin, B. Mosedale, *Nature* 283, 666 (1980); E. S. Cathcart *et al.*, *Lab. Invest.* 54, 26 (1986).
4. N. A. Andriopoulos *et al.*, *Arthritis Rheum.* 19, 613 (1976); D. E. Trentham, R. A. Dynesius, R. E. Rocklin, J. R. David, *N. Engl. J. Med.* 299, 327 (1978); A. Tarkowski, L. Klareskog, H. Carlsten, P. Herberts, W. J. Koopman, *Arthritis Rheum.* 32, 1087 (1989).
5. P. M. Brooks, *Lancet* 341, 286 (1993).
6. P. J. Higgins and H. L. Weiner, *J. Immunol.* 140, 440 (1988).
7. D. Bitar and C. C. Whitacre, *Cell. Immunol.* 112, 364 (1988); C. C. Whitacre, I. E. Gienapp, C. G. Orosz, D. Bitar, *J. Immunol.* 147, 2155 (1991).
8. R. B. Nussenblatt *et al.*, *J. Immunol.* 144, 1689 (1990).
9. O. Lider, M. B. Santos, C. S. Y. Lee, P. J. Higgins, H. L. Weiner, *ibid.* 142, 748 (1989).
10. A. Miller, O. Lider, H. L. Weiner, *J. Exp. Med.* 174, 791 (1991); S. J. Khoury, W. W. Hancock, H. L. Weiner, *ibid.* 176, 1355 (1992); A. Miller, O. Lider, A. Roberts, M. B. Sporn, H. L. Weiner, *Proc. Natl. Acad. Sci. U.S.A.* 89, 421 (1992).
11. Z. J. Zhang, L. Davidson, G. Eisenbarth, H. L. Weiner, *Proc. Natl. Acad. Sci. U.S.A.* 88, 10252 (1991).
12. H. L. Weiner *et al.*, *Science* 259, 1321 (1993).
13. C. Nagler-Anderson, L. A. Bober, M. E. Robinson, G. W. Siskind, G. J. Thorbecke, *Proc. Natl. Acad. Sci. U.S.A.* 83, 7443 (1986); H. S. G. Thompson and N. A. Staines, *Clin. Exp. Immunol.* 64, 581 (1986).
14. Z. J. Zhang, C. S. Y. Lee, O. Lider, H. L. Weiner, *J. Immunol.* 145, 2489 (1990).
15. Native type II collagen, isolated from sternal cartilage of chicks rendered lathyritic by administration of β-aminoproprionitrile (*2*), was used to treat the first five subjects in the phase I pilot study. Subsequent patients in the pilot trial and in the double-blind study received type II collagen purified from nonlathyritic chicken sternal cartilage by the identical technique (*2*) and obtained from Genzyme (Boston, MA). Preparations were analyzed for purity by standard biochemical methods (*2, 35*) and tested for arthritogenicity and toxicity in rats (*2*) with findings of batch-to-batch equivalency. Collagen was stored in a lyophilized state (*2*) at −20°C with desiccant. The protein was solubilized in 0.1 M acetic acid for ~12 hours at 4°C, sterilized by membrane filtration, and aliquoted into individual 1.0-ml doses in sterile tubes. Tubes sufficient for ~2 weeks of treatment were delivered on ice to patients and maintained under refrigeration until use. For oral administration, the 1.0-ml aliquot was added to 4 to 6 ounces (118 to 177 ml) of cold orange juice and the mixture drunk immediately. Orange juice provided an additional acid vehicle to inhibit precipitation of collagen and masked the taste of acetic acid. All dosing occurred in the morning on an empty stomach at least 20 min before breakfast or ingestion of other fluids. Smoking was not permitted during this interval.
16. M. E. Weinblatt *et al.*, *N. Engl. J. Med.* 312, 818 (1985); K. L. Sewell *et al.*, *Arthritis Rheum.*, in press.
17. R. S. Pinals, A. T. Masi, R. A. Larsen, *Arthritis Rheum.* 24, 1308 (1981).
18. The following requirements determined eligibility: (i) American Rheumatism Association (ARA) criteria for classic or definite rheumatoid arthritis (*16*); (ii) onset of the disease at age 16 or older; (iii) age at least 18 years; (iv) ARA functional class II or III (*28*); (v) signs or symptoms of synovitis unresponsive to at least one immunosuppressive (Table 1); and (vi) severe active disease defined by at least three of the following: ≥nine painful or tender joints, ≥six swollen joints, ≥45 min of morning stiffness, or ≥28 mm/hour ESR. Exclusion criteria included a degree of structural joint damage not amenable to physical rehabilitation if inflammation subsided after treatment or a serious concurrent medical problem. Some patients ($n = 39$) represented referrals for treatment of refractory disease by rheumatologists outside Boston; others ($n = 10$) had received experimental therapy for rheumatoid arthritis in the past.
19. The study was approved by the Beth Israel Hospital Committee on Clinical Investigations and conducted under an investigator-initiated Investigational New Drug (IND) permit from the U.S. Food and Drug Administration.
20. Because of the possibility that patients would receive ineffective therapy or a placebo, study medication was begun immediately after the patient discontinued immunosuppressive drugs (Table 1); patients receiving parenteral gold were not entered because prolonged carryover effects could influence the outcome. Patients remained on their NSAID, prednisone dose (≤10 mg/day), or both, during the 3-month treatment period. NSAID substitution, increases in NSAID or prednisone dose, or initiation of any other antirheumatic therapy with the exception of analgesic agents and intraarticular steroids represented protocol violations. If applicable, patients were requested to practice contraception.

21. A biostatistician (E.J.O.) randomized each patient to either the active or placebo treatment group in blocks of six, stratified by functional class (*28*) severity.
22. The placebo consisted of 1.0-ml doses of 0.1 M acetic acid subjected to membrane filtration.
23. Three investigators (D.C., C.L., and K.L.S.) obtained the randomization and prepared medication but did not have access to clinical data. No unblinding occurred.
24. Conventional instruments were used to measure RA activity (*16*). Assistive devices were permitted for walk times. The clinical investigator cared for the patients during the trial and was responsible for safety monitoring. Laboratory safety assessment was performed immediately before randomization and at 2, 4, 8, and 12 weeks thereafter. The assessment comprised a complete blood count, differential and platelet count, liver and renal function tests, prothrombin and partial thromboplastin times, urinalysis, and ESR. HLA typing was performed for alleles of the A, B, C, and DR/DQ loci (*36*). Serum immunoglobulin M (IgM) rheumatoid factor titers were determined by nephelometry and IgG antibody titers to native type II collagen (expressed as $-\log_2$) by enzyme-linked immunosorbent assay (*37*) immediately before and at the end of collagen or placebo administration.
25. Before unblinding, decisions were made concerning the analysis of five subjects (8%) that failed to complete the study. One was noncompliant and withdrew for personal reasons on day 40 after only a baseline examination. This patient was excluded from analysis and had been randomized to collagen. Four discontinued their study medication before the end of the 3-month treatment because of worsening arthritis. They were assigned the worst score in the sample for the remainder of the study and included in the analyses. All four had been randomized to placebo. One protocol violation occurred with a patient who increased the daily dose of prednisone from 5 mg to 10 mg just before month 2. Because the patient continued to do poorly and the 10-mg dose was consistent with eligibility requirements, the patient was included in the analyses; the patient had been randomized to collagen. No steroid injections or other problems with compliance occurred.
26. Comparisons between collagen- and placebo-treated patients were performed with the Wilcoxon rank-sum test for continuous measures (such as the number of swollen joints), the Fisher's exact test for dichotomous measures (such as narcotic usage), and the χ^2 trend test for functional class and patient and physician assessments. All measured end points such as the number of swollen joints were compared with entry values before testing; qualitative measures, such as patient and physician assessments and functional class, are presented and analyzed without adjustment for baseline responses. The Student's paired *t* test was used to assess whether changes in the collagen group represented significant improvements over baseline values. Reported *P* values are two-sided.
27. Complete resolution is a more rigorous extension of RA remission criteria (*17*), preformulated because of the magnitude of improvement in some patients in the initial trial, and is defined by the following conditions: no swollen or tender joints, no morning stiffness or afternoon fatigue, absent arthritis on physician and patient appraisals, functional class I status, and normal ESR (<28 mm/hour) while off prednisone.
28. O. Steinbrocker, C. H. Traeger, R. C. Batterman, *J. Am. Med. Assoc.* 140, 659 (1949).
29. Rather than assigning the placebo patients who withdrew from the trial the worst observed value (*25*), they were given the value from their last visit. Because one of the four dropped out before the 1-month follow-up, that patient was removed from all analyses, reducing the sample size to 28 collagen and 30 placebo patients. By this analysis, the number of tender joints, joint-tenderness index, walk time, patient assessment of severe or very severe disease, and analgesic use was significantly ($P \leq 0.05$) improved in the collagen group compared with the placebo group.
30. H. J. Williams *et al.*, *Arthritis Rheum.* 31, 702 (1988).
31. Analysis of variance showed no significant interaction between treatment effectiveness (as measured by changes in the number of swollen joints, tender joints, or walk time) and any characteristic in Table 1.
32. R. Nussenblatt, personal communication.
33. A. Friedman and H. L. Weiner, *J. Immunol.* 150, 4A (1993); H. L. Weiner *et al.*, *Ann. Rev. Immunol.*, in press.
34. A. Al-Sabbagh, A. Miller, R. A. Sobel, H. L. Weiner, *Neurology* 42 (suppl. 3), 346 (1992).
35. S. M. Helfgott, R. A. Dynesius-Trentham, E. Brady, D. E. Trentham, *J. Exp. Med.* 162, 1531 (1985).
36. G. M. Kammer and D. E. Trentham, *Arthritis Rheum.* 27, 489 (1984).
37. S. M. Helfgott *et al.*, *Lancet* 337, 387 (1991).
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Tyrosine Phosphorylation of DNA Binding Proteins by Multiple Cytokines

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Interferon-α (IFN-α) and IFN-γ regulate gene expression by tyrosine phosphorylation of several transcription factors that have the 91-kilodalton (p91) protein of interferon-stimulated gene factor–3 (ISGF-3) as a common component. Interferon-activated protein complexes bind enhancers present in the promoters of early response genes such as the high-affinity Fcγ receptor gene (FcγRI). Treatment of human peripheral blood monocytes or basophils with interleukin-3 (IL-3), IL-5, IL-10, or granulocyte-macrophage colony-stimulating factor (GM-CSF) activated DNA binding proteins that recognized the IFN-response region (GRR) located in the promoter of the FcγRI gene. Although tyrosine phosphorylation was required for the assembly of each of these GRR binding complexes, only those formed as a result of treatment with IFN-γ or IL-10 contained p91. Instead, complexes activated by IL-3 or GM-CSF contained a tyrosine-phosphorylated protein of 80 kilodaltons. Induction of FcγRI RNA occurred only with IFN-γ and IL-10, whereas pretreatment of cells with GM-CSF or IL-3 inhibited IFN-γ induction of FcγRI RNA. Thus, several cytokines other than interferons can activate putative transcription factors by tyrosine phosphorylation.

Nuclear or whole-cell extracts prepared from human monocytes incubated with either IFN-γ or IFN-α contain a protein or proteins (FcRFγ) that specifically recognize the GRR in the promoter of the high-affinity immunoglobulin G Fc receptor gene (*1–3*). Within the FcRFγ complex is a 91-kD tyrosine-phosphorylated protein that is a component of the ISGF-3 transcription complex, which causes IFN-α–stimulated expression of early response genes (*2–4*). Because the peripheral blood monocyte is a critical target cell for IFN-α, IFN-γ, and other cytokines, experiments were done to determine whether any cytokines other than the interferons might induce the formation of FcRFγ. Whole-cell extracts were prepared from monocytes incubated with various cytokines for 15 min at 37°C and analyzed by electrophoretic mobility–shift assays (EMSAs) with a ^{32}P-labeled oligonucleotide corresponding to the GRR (Fig. 1A) (*5*). Untreated cells showed no formation of FcRFγ, whereas extracts prepared from monocytes treated with IL-3 or GM-CSF contained GRR binding complexes that migrated with a mobility different than that of the FcRFγ (Fig. 1A) complex observed after IFN-γ activation. In contrast, IL-10 activated the formation of a GRR binding complex with a mobility similar to that of FcRFγ. Other cytokines that have effects on monocytes—IL-1, IL-2, IL-6, tumor necrosis factor (TNF), monocyte colony-stimulating factor (M-CSF), and lipopolysaccharide—showed no formation of GRR binding complexes.

Binding of FcRFγ and the complexes activated by GM-CSF treatment of monocytes was inhibited by addition of excess unlabeled GRR (Fig. 1B), but not by addition of an unlabeled oligonucleotide corresponding to the IFN-γ activation sequence (GAS) within the promoter of the guanylate-binding protein gene (Fig. 1B) (*6*). The complexes induced by treatment of monocytes with IL-3 and IL-10 showed similar binding specificities (*7*). When the GAS oligonucleotide was used as a probe,

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ORIGINAL PAPER

Use of Undenatured Type II Collagen in the Treatment of Rheumatoid Arthritis

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ABSTRACT: Rheumatoid arthritis is a debilitating chronic disease that lacks an effective treatment. It is the leading cause of disability in the United States. Rheumatoid arthritis is an inflammatory response believed to involve T cells reacting to an antigen within the joints and articular cartilage. Over-the-counter pain relievers and anti-inflammatory medications, such as aspirin, acetaminophen, and ibuprofen, are commonly used for preventive measures, but these products only treat the symptoms, not the cause, and may also produce serious side effects. A growing body of evidence indicates that type II collagen is a major structural protein responsible for tensile strength and toughness in the cartilege and also a potential autoantigen in people who have rheumatoid arthritis. If the activity of T cells that release joint-destroying factors could be reduced, outcomes for patients with rheumatoid arthritis could be improved. One method of achieving this is termed *oral tolerance*, a concept that is proving useful in the treatment of autoimmune diseases. Oral tolerance describes a state of immune hyporesponsiveness following the oral ingestion of a protein. It is, therefore, a method by which a peripheral immune tolerance (down regulation) to a particular antigen may be induced by presenting specific amounts of that antigen to the gastrointestinal system. Several clinical studies have demonstrated the effectiveness and usefulness of undenatured collagen II in attenuating the symptoms of rheumatoid arthritis with no serious adverse effects. Thus its administration may demonstrate therapeutic efficacy by inducing oral tolerance for the treatment of this disease.

Introduction

Arthritis is one of the most prevalent chronic health problems in the United States, affecting nearly 43 million people.[1] Although it is often thought of as a disease that predominantly affects the elderly, it is the number 1 cause of disability affecting those over the age of 15. In fact, more than half of those affected by arthritis are under the age of 65, and almost 300,000 of those affected are children.[1] Each year arthritis is responsible for 44 million outpatient visits and almost 1 million hospitalizations, and it is second only to heart disease in terms of its effect on days lost from work.[2] As might be imagined from these statistics, the toll that arthritis takes on the healthcare industry is substantial, costing the United States approximately $65 billion each year in health-related expenses. Unfortunately, the incidence of arthritis does not appear to be decreasing, and by the year 2020 the Centers for Disease Control (CDC) predicts that almost 60 million Americans will suffer from some form of this disease.

While the term *arthritis* may bring to mind a simple condition characterized by painful joints and difficulty performing certain tasks, it actually encompasses more than 100 different diseases. Of these different forms of arthritis, osteoarthritis (OA) and rheumatoid arthritis (RA) are the most common.

Osteoarthritis. OA currently affects 20 million Americans. It is a degenerative joint disease in which the cartilage covering the ends of bones deteriorates, resulting in pain, stiffness, and loss of movement.[3,4] This form of arthritis generally begins after the age of 40 and develops slowly over many years.[5] People usually report pain beginning in joints on only one side of the body, in contrast to RA. While inflammation may be present, joint pain in OA is typically not accompanied by the amount or severity of inflammation observed in those with RA. Weight-bearing joints such as the knee and hip tend to be more affected than non-weight-bearing joints such as the elbow or shoulder. The general feeling of sickness that can accompany other forms of arthritis does not usually accompany OA.

Rheumatoid Arthritis. RA is not a "new" disease. One of the first descriptions of a disease resembling RA can be found in the *Charaka Samhita*, a medical text from India that dates back as far as 500 BC. Another ancient reference to the disease dates to 100 BC: the Roman Scribonius Largus described a polyarthritic condition occurring mainly in elderly women that closely resembled what we now understand to be RA. Rheumatoid arthritis currently affects approximately 2 million Americans and about 1% of the world's population. However, the pathology and progression of RA is somewhat different from that of OA.[6,7] It often develops

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suddenly, within weeks or months, and generally begins between the ages of 25 and 50. Non-weight-bearing joints such as the hands, shoulders, and elbows are usually affected bilaterally, and a significant amount of redness, tenderness, swelling, and inflammation is often present. RA often results in a feeling of sickness and fatigue and may be accompanied by weight loss as well as fever.[6] Morning joint stiffness lasting for an hour or longer is relatively common. Subcutaneous nodules that form over bones may also be present. Interestingly, 3 times as many women as men are afflicted with rheumatoid arthritis. Some patients will experience a monocyclic course of the disease that may abate within 2 years, while others will experience a polycyclic, or progressive, course. Of all the forms of arthritis, RA tends to be one of the most serious and disabling; 16% of those who have had the disease for 12 or more years become completely disabled. Lifespan has been shown to be shortened by approximately 7 years in men and 3 years in women, which is equivalent to the increased mortality observed in those with diabetes and Hodgkin's disease.

Etiology

While RA is classified as an autoimmune disease, the exact causes that result in its development remain a mystery. What is known is that many different and complex factors are involved. While some cases of OA may be the result of years of "wear and tear" on joint structures, other forms can be traced to an injury, infection, or metabolic disorder.[6,8] RA, however, does not result from overuse of a joint or from injury, but rather from an autoimmune problem in which the body attacks and damages its own tissue. The damage that occurs in RA appears to be propagated by cytokines secreted by T cells in response to certain autoantigenic stimuli within the joint.[9,10] Various immunological factors are involved, including but not limited to CD4-inducer lymphocytes, CD4 memory cells, macrophages, neutrophils, and tumor necrosis factor.[11,12] One of the most likely candidates for this autoantigenic stimulus is the collagen component of cartilage, specifically type II collagen. Some researchers believe that an infection may trigger the initial inflammation in a joint through molecular mimicry or other mechanisms, which in turn initiates the autoimmune response. Genetics may also play a role, as may other factors, including stress.

The cartilage in joints allows for flexibility and motion and provides a cushion against the impact of various forces on the bone.[13] The detailed structure of cartilage is complex but can be simplified into 2 major components: collagen and proteoglycans. Proteoglycans are large protein molecules attached to large carbohydrate chains called glycosaminoglycans.[13] These proteoglycans help to provide a matrix in which the collagen as well as water can coexist. There are a number of different types of collagen, but type II accounts for the major part of cartilage. It is composed of 3 identical chains (termed α-1 chains) that form a triple helix.[13-15] This interconnected network of collagen and proteoglycans is crucial in maintaining joint flexibility and resistance to stress and fracture. In RA, autoantigenic responses, most likely triggered by type II collagen, ultimately result in the progressive inflammation, pain, and destruction characteristic of this disease.[16]

Regardless of the initial cause, a progressive degeneration of the structure and function of the joint takes place, making normal activities of life increasingly difficult. In the case of RA, the body is unable to recognize its collagen as normal and in turn attacks it as if it were a foreign invader.[10,17] A novel approach to treatment, termed *oral tolerance*, in which small amounts of type II collagen are presented to the gastrointestinal tract, has been the focus of significant positive scientific research.[18] What is achieved is a down regulation of the body's ability to destroy its own collagen, resulting in improvement in symptoms and slowing the progression of the disease. However, to fully appreciate the use of oral tolerance in the treatment of RA, it is important to understand the typical treatment options currently in use.[19]

Current Treatment Options

The treatment options for those with rheumatoid arthritis are typically nonsteroidal anti-inflammatory drugs (NSAIDs), alone or in combination with what are known as disease-modifying antirheumatic drugs (DMARDs). As is well known, chronic use of NSAIDs, especially in the elderly, is linked to numerous side effects, including gastrointestinal bleeding and renal malfunction.[20] Even the newer generation of COX-II inhibitors such as rofecoxib (a furanone derivative),[21] celecoxib (a 1,5-diaryl substituted pyrazole),[22] and infliximab (a monoclonal antibody) are not without their own problems.[23,24] While these drugs do reduce inflammation, they do not address the underlying causes of the arthritis and therefore cannot alter the progression of the disease. Furthermore, rofecoxib and celecobix have been contraindicated for use by patients suffering from hypersensitivity, asthma, urticaria, or allergic reactions. All of them can cause bleeding, ulceration, perforation of the stomach and intestines, and anaphylactoid reactions. In addition, rofecoxib has recently been associated with a possible increased risk of heart attack and stroke.[20] Prolonged use of these newer COX-II inhibitors in the elderly can result in side effects similar to those seen with traditional NSAIDs. DMARDs attempt to address the underlying pathology of RA more thoroughly by slowing

the progression of the pathology. One of the components of this disease, in addition to inflammation, is microvascular injury coupled with the formation of new capillaries. Many of the DMARDs attempt to inhibit the formation of new capillaries as well as address the underlying inflammation. This category of drugs includes azathioprine, corticosteroids, gold, hydroxychloroquine, methotrexate, sulfasalazine, and a number of newer medications such as leflunomide and etanercept. While these medications have been shown to offer clinical improvement to those with rheumatoid arthritis, they can also be associated with significant toxicity and side effects, including myelosuppression, lymphoproliferative disorders, macular damage, thrompocytopenia, osteoporosis, hyperglycemia, and hepatotoxicity. Another factor that should be taken into account is cost: the monthly cost for etanercept and infliximab, both of which must be injected, can be more than $1000. Also, according to a recent report by the FDA, Remicade (infliximab) has been associated with tuberculosis infection, nerve damage, and risk of cancer lymphoma in patients. In severe cases of joint damage, surgery is often necessary, but this is also costly and involves a lengthy recovery time.[20]

Methylsulfonylmethane, chondroitin, and glucosamine, widely used for treatment of OA,[25] are known to help rebuild proteoglycans and reduce inflammation but are unable to help in the process of inactivating "killer" T cells to ameliorate rheumatoid arthritis.

Oral Tolerance

When the immune system is functioning properly, it recognizes and identifies foreign substances in order to help eliminate them from the body.[10,18] One type of immune cell that is particularly important in this process is the T cell, which can be classified in a number of different categories, depending on function. "Helper" T cells have the function of releasing factors that help increase or decrease the immune response. These have been further classified into Th-1 and Th-2 subsets. Th-1 cells amplify proinflammatory responses; Th-2 cells limit such responses. "Killer" T cells attack and destroy antigens. The B cell is also crucial to the functioning of the immune system, as it is responsible for the production of antibodies. In a normal individual, the immune system does not seek out and destroy healthy tissue due in part to the fact that T cells that have specificity for antigens on normal tissue are either suppressed or destroyed prior to being released into circulation. In the case of rheumatoid arthritis, however, T cells with self-antigens for type II collagen are not properly destroyed or suppressed, resulting in the damage that is a characteristic hallmark of this disease.

By decreasing the activity of T cells that are releasing joint-destroying factors, the outcome for patients with RA can be improved, and one such method to achieve this is oral tolerance. The concept of oral tolerance has existed since 1911, and traditional medical literature is filled with papers describing this mechanism and how it might benefit those with autoimmune diseases.[10]

Recent studies have shown that small doses of type II collagen derived from chicken cartilage produce oral tolerance and work with the immune system to prevent the body from attacking its joints.[8,18]

Oral tolerance can be induced by 2 major mechanisms, bystander suppression and clonal anergy, depending on the dose of antigen that is presented. Throughout the small intestine, there are patches of gut-associated lymphoid tissue (GALT). Within the GALT can be found tissue that consists of nodules (Peyer's patches) that contain organized assemblages of T and B lymphocytes, macrophages, and dendritic cells and are the primary area within the gastrointestinal tract where immune responses are generated. This immune tissue is designed to protect the host from ingesting pathogens as well as to prevent the host from reacting to ingested proteins. In fact, scientists have attempted to use the GALT as a route for administering vaccines but have been deterred by systemic hyporesponsiveness. Nonetheless, the generation of immune responses within the GALT is the primary mechanism by which orally ingested proteins can suppress systemic immunity.

Bystander Suppression

This form of oral tolerance is achieved by presenting small amounts of antigen to the GALT, which in turn generates a T-cell response. After the antigen (in this case, type II collagen) is consumed, regulatory Th2 and Th3 cells migrate from the GALT through the lymphatic system and then into peripheral circulation. When they encounter an antigen similar to that which was ingested, they secrete cytokines, including transforming growth factor-beta, interleukin-4, and interleukin-10, that result in the down regulation of activated helper Th1 cells. It is these activated helper T cells that are, in part, involved in producing the inflammation and destruction of collagen in RA. If this activity against healthy collagen can be decreased, the progression of the disease can be altered. It should be noted that the oral antigen does not need to enter the systemic circulation in order to induce a response, as the regulatory T cells are induced as a result of the interaction between the antigen and the GALT.[10]

Clonal Anergy

Another mechanism by which an orally administered protein can induce a down regulation of an immune response is via a mechanism called clonal anergy.[10] This

situation results from the ingestion of high doses of an antigen, which in turn induces a state of unresponsiveness from overactive Th1 cells. These cells are not deleted but are rendered incapable of responding to a specific antigen. In essence, they are turned off, or "anergized," and will no longer recognize the antigen as a target for destruction.[10]

Clinical Studies

Via the mechanism of oral tolerance, type II collagen has been studied for its ability to benefit those with RA. This makes sense, because type II collagen is the most abundant structural protein present in cartilage. Numerous animal models of arthritis have demonstrated significant benefit from orally administered, native (undenatured) type II collagen. Its administration has been able to suppress almost all experimentally inducible forms of RA in animals, including antigen-induced arthritis, adjuvant arthritis, type II collagen-induced arthritis, streptococcal cell-wall arthritis, and silicone-induced arthritis. These impressive results led to the investigation of native type II collagen supplementation in humans with RA. In 1993, an open-label pilot trial and a phase II trial in humans were conducted at Harvard Medical School.[8] In the pilot trial, 10 patients diagnosed with RA had their immunosuppressive and disease-modifying drugs discontinued and were given 0.1 mg of native type II collagen daily for 1 month, followed by 0.5 mg of native type II collagen for the next 2 months. Six of the 10 subjects experienced a significant improvement (defined as >50% compared with baseline) in swollen and tender joint counts, as well as morning stiffness, 50-foot walk time, grip strength time, and erythrocyte sedimentation rate. One subject who had previously been treated with methotrexate experienced complete remission, which continued for 26 months. No adverse effects were noted.[8] Because of these observed improvements, a placebo-controlled phase II follow-up trial was performed consisting of 60 subjects with severe, active RA. Participants were randomly assigned to groups taking either a placebo or a daily dose of 0.1 mg native type II collagen for 1 month, then 0.5 mg for 2 months. At 1, 2, and 3 months, the collagen group experienced significant improvement ($P < 0.05$) in the number of swollen joints, the number of painful and tender joints ($P = 0.06$ at 2 months), and 50-foot walk time. Four patients in the collagen group, compared with no patients in the placebo group, experienced complete remission of the disease. One of the most notable findings was the lack of side effects as a result of the treatment, an important issue given the side effects that can be present with various DMARDs and NSAIDs. Importantly, a recent independent report has also confirmed the effectiveness of type II collagen in juvenile RA,[26] a disease affecting almost 300,000 children. Ten patients between the ages of 8 and 14 years who had active RA were treated orally with type II collagen for 3 months. Eight of the 10 patients had a reduction in both swollen and tender joints at the end of 3 months. One patient in this study also achieved complete remission. It was concluded that oral treatment with native type II collagen may be a safe and effective form of treatment for juvenile RA.[26]

A fourth study[27] of native type II collagen supplementation in RA reported significant improvement in subjects who met Paulus criteria (morning stiffness, joint tenderness, joint swelling, and erythrocyte sedimentation rate). After 24 weeks, 39% of those taking type II collagen versus 19% taking placebo experienced significant improvement. While 19% may appear to be a large response in the placebo group, it is not unusual to observe this type of response in studies of arthritis. The impressive finding was the high degree of improvement in the group treated with undenatured, type II collagen as compared to the group taking the placebo. An interesting observation in this study was that subjects with a presence of serum IgA and IgG antibodies to collagen at the beginning of the study had a significantly better response to treatment than those lacking such antibodies.

In a fifth double-blind, placebo-controlled study performed in Germany, 90 subjects with early RA were divided into groups receiving daily doses of 1 mg collagen, 10 mg collagen, or placebo.[28] At the end of the study, 3 patients in the 10 mg group, 1 patient in the 1 mg group, and no patients in the placebo group had experienced marked improvement. While these results may not appear very impressive, the authors were surprised by the degree of benefit given the small subset of patients. In another German study,[16] daily doses of 1 mg or 10 mg of undenatured type II collagen resulted in reduced type II collagen antibody titres in patients showing a clinical response. This study also suggested that 10 mg was a more effective dose than 1 mg.[16] These studies provide the basis and rationale for the use of native type II collagen as a safe and effective modality of treatment for those suffering from RA.

The Importance of a Native (Undenatured) Form of Collagen

To confer oral tolerance, type II collagen must be used in its *undenatured*, 3-dimensional, triple-helical structure.[10,29] Unfortunately, most products on the market containing type II collagen do not contain the undenatured form. In these products it has undergone harsh chemical or high-temperature manufacturing procedures which denature it, thus rendering it inactive and incapable of eliciting an immune response once administered. In fact, no peer-reviewed studies exist to support

the use of *denatured* type II collagen in RA, and one study has shown denatured type II collagen to have no impact on the severity of the disease.[29] In order to insure that undenatured type II collagen is present, highly sensitive ELISA assays must be performed to confirm that the collagen is biologically active.

Source of Undenatured Type II Collagen

It is well understood that type II collagen can be obtained from all types of animals, including mice, rats, chickens, pigs, and dogs, as well as from humans. However, an ideal commercial source would be to obtain it in a cost-effective way from animals housed and maintained in a germ-free environment. Chickens raised in a controlled environment with ambient temperature and purified air, free from bacteria, viruses, fungi, and other microorganisms, are currently the best source of commercial undenatured type II collagen.

Dose

Clinical studies support the use of native, undenatured type II collagen and recommend that it be taken with water at bedtime. Furthermore, studies have shown that small doses (typically 10 mg or less) derived from chicken cartilage work with the human immune system to promote healthy joints and improve mobility and flexibility, as well as attenuating the symptoms of RA. The ideal situation is to ingest undenatured collagen II on an empty stomach when the acid content in the stomach is low. Generally, protein absorption in a human body may take from 4 to 8 hours.

Potential Use of Undenatured Type II Collagen in Osteoarthritis

Therapeutic interventions that work rapidly for RA, such as NSAIDs or cortisone injections, are also palliative for OA. Unfortunately, drugs that work rapidly for OA do not, in general, provide sufficient reduction of inflammation or pain relief on their own in rheumatoid arthritis. OA therapies in this category include NSAIDs, hylan g-f 20, and most probably glucosamine hydrochloride and chondroitin sulfate. Presumably, this dichotomy relates to the much more substantial degree of inflammation present in RA versus OA.

OA is a wear and tear phenomenon usually associated with aging; the disease progresses with rigorous exercise when muscles and bones are already weakened due to aging (exercise also causes muscle and bone damage in aged patients with RA). It is also characterized by an inflammatory synovial response that leads to joint wear and tear.[30] As RA will effectively cause gradual deterioration and inflammation of certain joints due to immune disorders, OA will cause wear and tear due to the normal aging process and an increase in enzymatic activity. In the absence of significant and disfiguring inflammation (which is characteristic of rheumatoid arthritis), wear and tear activity may be misdiagnosed as OA rather than RA and treated accordingly. In some cases, OA is added as an additional diagnosis simply because wear and tear and aging persists and exists normally. The biochemical markers associated with OA inflammation, such as various cytokines (interleukin-4 and interleukin-10), tumor necrosis factor-alpha, and interferons are also associated with RA inflammation.[11,30,31] Therefore, therapies used to treat RA inflammation are also used to treat severe OA inflammation. Earlier research demonstrates that type II collagen suppresses T-cell-mediated inflammation, which is characterized by cytokines interleukin-4 and interleukin-10 and is seen in the synoviums of both OA and RA patients. Another benefit of type II collagen is that it contains small amounts of glucosamine and chondroitin, which are good for joint mobility and flexibility. In light of these facts, it may be postulated that undenatured type II collagen may also provide benefit to a significant population of OA patients as well as those with RA.

Conclusion

Finding an effective cure for RA is a major challenge for health professionals. Over-the-counter pain relievers, NSAIDS and other anti-inflammatory drugs, and monoclonal antibody and COX-II inhibitors have major adverse side effects, including liver disease, gastritis, vomiting, cardiovascular dysfunctions, and, possibly, tuberculosis. Futhermore, infliximab, rofecoxib, and celecoxib are very expensive drugs for regular use. Another expensive alternative is surgery, which involves a long recovery time. Several human clinical trials have demonstrated the effectiveness and usefulness of undenatured type II collagen in significantly reducing the painful symptoms of RA with no adverse side effects.

References

1. Helmick CG, Lawrence RC, Pollard RA, Lloyd E, Heyse SP. Arthritis and other rheumatic conditions: who is affected now, will be affected later? *Arthritis Care Res.* 1995;8:203-211.
2. Centers for Disease Control and Prevention. Prevalence of disability and associated health conditions—United States, 1991-1992. *MMRW Morb Mortal Wkly Rep.* 1994;4341:737-739.
3. Peat G, McCarney R, Croft P. Knee pain and osteoarthritis in older adults: a review of community burden and current use of primary health care. *Ann Rheum Dis.* 2001;60:91-97.
4. Bellamy N, Carr A, Dougados M, Shea B, Wells G. Towards a definition of "difference" in osteoarthritis. *J Rheumatol.* 2001;28:427-430.
5. Goldring MB. Osteoarthritis and cartilage: the role of cytokines. *Curr Rheumatol Rep.* 2000;2:459-465.
6. Okada Y. Pathoetiology of chronic rheumatoid arthritis—from inflammation to bone destruction. 3. From the viewpoint of pathology. *Nippon Naika Gakkai Zasshi.* 2000;89:2072-2080.
7. Aceves-Avila FJ, Medina F, Fraga A. The antiquity of rheumatoid arthritis: a reappraisal. *J Rheumatol.* 2001;28:751-757.
8. Trentham DE, Dynesius-Trentham RA, Orav EJ, et al. Effects of oral admin-

istration of type II collagen on rheumatoid arthritis. *Science*. 1993;261:1727-1730.
9. Wollheim FA. Approaches to rheumatoid arthritis in 2000. *Curr Opin Rheumatol*. 2001;13:193-201.
10. Weiner HL. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol Today*. 1997;18:335-343.
11. Nanki T, Lipski PE. Cytokine, activation marker, and chemokine receptor expression by individual CD4+ memory T cells in rheumatoid arthritis synovium. *Arthritis Res*. 2000;2:415-423.
12. Feldmann M, Maini RN. Anti-TNFα therapy of rheumatoid arthritis: What have we learned? *Ann Rev Immunol*. 2001;19:163-196.
13. Mayne R. Cartilage collagens: what is their function, and are they involved in articular disease? *Arthritis Rheum*. 1989;32:241-246.
14. Moller HJ. Connective tissue markers of rheumatoid arthritis. *Scand J Clin Lab Invest*. 1998;58:269-278.
15. Cook AD, Rowley MJ, Mackay IR, Gough A, Emery P. Antibodies to type II collagen in early rheumatoid arthritis: correlation with disease progression. *Arthritis Rheum*. 1996;39:1720-1727.
16. Gimsa U, Sieper J, Braun J, Mitchison NA. Type II collagen serology: a guide to clinical responsiveness to oral tolerance. *Rheumatol Int*. 1997;16:237-240.
17. Condemni JJ. The autoimmune diseases. *JAMA*. 1992;268:2882-2892.
18. Trentham DE. Oral tolerization as a treatment of rheumatoid arthritis. *Rheum Dis Clin North Am*. 1998;24:525-536.
19. Matteson EL. Current treatment strategies for rheumatoid arthritis. *Mayo Clin Proc*. 2000;75:69-74.
20. *Physicians Desk Reference*. 52nd ed. Montvale, NJ: Medical Economics Company, Inc; 1998.
21. Matheson AJ, Figgilt DP. Rofecoxib: a review of its use in the management of osteoarthritis, acute pain and rheumatoid arthritis. *Drugs*. 2001;61:833-865.
22. Davies NM, Gudde TW, de Leeum MA. Celecoxib: a new option in the treatment of arthropathies and familial adenomatous polyposis. *Expert Opin Pharmacother*. 2001;2:139-152.
23. Schuna AA, Megeff C. New drugs for the treatment of rheumatoid arthritis. *Am J Health Syst Pharm*. 2000;57:225-234.
24. Infante R, Lahita RG. Rheumatoid arthritis: new disease-modifying and anti-inflammatory drugs. *Geriatrics*. 2000;55:30-32.
25. Walker-Bone K, Javid K, Arden N, Cooper C. Glucosamine and chondroitin may help in osteoarthritis. *BMJ*. 2001;322:673.
26. Barnett ML, Combitchi D, Trentham DE. A pilot trial of oral type II collagen in the treatment of juvenile rheumatoid arthritis. *Arthritis Rheum*. 1996;39:623-628.
27. Barnett ML, Kremer JM, St. Clair EW, et al. Treatment of rheumatoid arthritis with oral type II collagen: results of a multicenter, double-blind placebo-controlled trial. *Arthritis Rheum*. 1998;41:290-297.
28. Sieper J, Kary S, Sorensen H, et al. Oral type II collagen treatment in early rheumatoid arthritis: a double-blind, placebo-controlled, randomized trial. *Arthritis Rheum*. 1996;39:41-51.
29. Nagler-Anderson C, Bober LA, Robinson ME, Siskind GW, Thorbecke GJ. Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen. *Proc Natl Acad Sci USA*. 1986;83:7443-7446.
30. Furuzawa-Carballeda J, Alcocer-Varela J. Interleukin-8, interleukin-10, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression levels are higher in synovial tissue from patients with rheumatoid arthritis than in osteoarthritis. *Scand J Immunol*. 1999;50:215-222.
31. van Roon JA, Lafeber FP, Bijlsma JW. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. *Arthritis Rheum*. 2001;44:3-12.

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Therapeutic Efficacy and Safety of Undenatured Type II Collagen Singly or in Combination with Glucosamine and Chondroitin in Arthritic Dogs

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ABSTRACT This investigation was undertaken to evaluate the therapeutic efficacy and safety of glycosylated undenatured type II collagen (UC-II) alone or in combination with glucosamine HCl and chondroitin sulfate in arthritic dogs. Twenty dogs divided into four groups (n = 5) were daily treated orally for 120 days: group I, placebo; group II, 10 mg UC-II; group III, 2,000 mg glucosamine + 1,600 mg chondroitin; group IV, UC-II (10 mg) + glucosamine (2,000 mg) + chondroitin (1,600 mg), followed by a 30-day withdrawal period. On a monthly basis, dogs were examined for overall pain, pain upon limb manipulation, and exercise-associated lameness. Serum samples were analyzed for markers of liver function (ALT and bilirubin) and renal function (BUN and creatinine). Body weight was also measured at a monthly interval. Dogs in group I exhibited no change in arthritic conditions. Dogs receiving UC-II alone showed significant reductions in overall pain within 30 days (33%) and pain upon limb manipulation and exercise-associated lameness after 60 days (66% and 44%, respectively) of treatment. Maximum reductions in pain were noted after 120 days of treatment (overall pain reduction, 62%; pain reduction upon limb manipulation, 91%; and reduction in exercise-associated lameness, 78%). The overall activity of the dogs in the UC-II supplemented with glucosamine and chondroitin group (group IV) was significantly better than the glucosamine + chondroitin-supplemented group (group III). Glucosamine and chondroitin alleviated some pain, but in combination with UC-II (group IV) provided significant reductions in overall pain (57%), pain upon limb manipulation (53%), and exercise-associated lameness (53%). Following withdrawal of supplements, all dogs (groups II to IV) experienced a relapse of pain. None of the dogs in any groups showed any adverse effects or change in liver or kidney function markers or body weight. Data of this placebo-controlled study demonstrate that daily treatment of arthritic dogs with UC-II alone or in combination with glucosamine and chondroitin markedly alleviates arthritic-associated pain, and these supplements are well tolerated as no side effects were noted.

KEYWORDS UC-II; Glucosamine; Chondroitin; Dog Arthritis; Pain Measurement

INTRODUCTION

Arthritis is a chronic disease that commonly affects large-breed dogs due to overweight/obesity, lack of exercise, physical injury, infection of joint surfaces, immune disorder, aging, or genetic predisposition. In particular, overweight and obesity in dogs can indirectly influence the degenerative joint disease process by increasing joint stress (Richardson et al. 1997). Dogs suffer most often with osteoarthritis than with rheumatoid arthritis (Hielm-Bjorkman et al. 2003). Arthritis is one of the most prevalent chronic health problems in the United States, affecting nearly 43 million people (Helmick et al. 1995). Although arthritis is often thought of as a disease that predominantly affects the elderly, it is the number one cause of disability affecting those over the age of 15. In fact, more than half of those affected by arthritis are under the age of 65, and almost 300,000 of those affected are children (Centers for Control and Prevention [CDC] 1994; Helmick et al. 1995; Trentham et al. 2001). Each year, arthritis is responsible for 44 million outpatient visits and almost 1 million hospitalizations, and is second only to heart disease in terms of its effect on disability from work (CDC 1994). As might be imagined from these statistics, the toll that arthritis takes on the health care industry is substantial, costing the United States approximately 65 billion dollars each year in health-related expenses. Unfortunately, the prevalence of arthritis does not appear to be decreasing, and by the year 2020 the CDC predicts that almost 60 million Americans will suffer from some form of arthritis.

Osteoarthritis is an inflammatory joint disease characterized by degeneration of the cartilage, hypertrophy of bone at the margins, and changes in the synovial membrane, and that eventually results in pain and stiffness of joints (Goldring 2000; Peat et al. 2001; Bellamy et al. 2001). Rheumatoid arthritis is a chronic disease characterized by inflammation, pain, swelling, and stiffness of multiple joints (Trentham et al. 1993; Okada 2000; Aceves-Avila et al. 2001). In either form of arthritis, dogs usually limp and are unable to move normally. Dog owners and veterinarians rarely notice the early warning signs of arthritis in dogs, since the dogs have the tendency to ignore soreness and discomfort until the arthritic signs are progressed significantly. Together, osteoarthritis and obesity cause a decreased quality of life for pets since joint pain is strongly associated with body weight (Richardson et al. 1997).

The present therapy of arthritis in dogs relies upon drugs that alleviate pain, control inflammation, and preserve ability to perform daily activity. Chronic use of cyclooxygenase (COX) inhibitors (nonsteroidal anti-inflammatory drugs [NSAIDs]) is linked to numerous side effects, including gastrointestinal (GI) bleeding and hepatic and renal dysfunction (Physician's Desk Reference [PDR] 1998; Muhlfeld and Floege 2005). Anti-inflammatory drugs, such as aspirin and ibuprofen, are nonspecific inhibitors of COX enzymes (both COX-I and COX-II), and they inhibit the production of inflammatory prostaglandins, thereby providing therapeutic effect, but they also inhibit the production of constitutive prostaglandins, causing severe side effects, such as severe GI bleeding (Matteson 2000). In the recent past, two commonly used drugs approved by the Food and Drug Administration (FDA) in arthritic dogs included Rimadyl (carprofen) and Deramaxx (deracoxib). Both Rimadyl and Deramaxx are NSAIDs. Rimadyl is not recommended for animals with known bleeding disorders and should not be used if a dog has pre-existing liver disease, inflammatory bowl disease, or a known tendency toward GI ulceration. Labrador Retrievers and other breeds are represented in the population that has experienced side effects or a fatal outcome from Rimadyl. Deramaxx (deracoxib), a COX-II inhibitor similar to Celebrex (celecoxib) and Vioxx (rofecoxib), prescribed as pain relievers for people, was withdrawn from the market in 2004, because of heart attack and stroke risk. According to a recent clinical study from the Mayo Clinic, other rheumatoid arthritic drugs such as Humira (adalimumab) or Remicade (infliximab) may cause serious infections or lead to the development of several kinds of cancer (Bongartz et al. 2006). Therefore, under the present circumstances, a safe therapy is needed for arthritic dogs.

In recent years, due to widespread availability of nutraceuticals, glucosamine and chondroitin sulfate were the two most commonly used supplements to ease the pain and discomfort of arthritis in dogs. Nutraceuticals are defined as functional foods, natural products, or parts of food that provide medicinal, therapeutic, or health benefits, including the prevention or treatment of disease. Glucosamine is an amino-monosaccharide precursor of the disaccharide unit of glycosaminoglycan, which is the building block of proteoglycans, the ground substance of articular cartilage (Paroli et al. 1991). Chondroitin sulfate is a part of a large protein molecule (proteoglycan) that

gives cartilage elasticity. Glucosamine is extracted from crab, lobster, or shrimp shells, and chondroitin is extracted from animal cartilage, such as tracheas and shark cartilage. In a recent pilot study, we found for the first time that daily administration of glycosylated undenatured type II collagen (40 mg of UC-II providing 10 mg/day) for 90 days significantly ameliorated the signs and symptoms of arthritis in dogs (DeParle et al. 2005). UC-II is a glycoprotein from chicken sternum cartilage. The presence of glycosylated "active" epitopes in the UC-II collagen matrix was confirmed by a validated ELISA method available from Chrondex, LLC (Arthrogen-CIA Capture "ELISA" test). Furthermore, electron microscopic analysis of UC-II was conducted to demonstrate the conformational integrity of the undenatured triple helical structure of a protein (Bagchi et al. 2002). Based on this study and other research (Bagchi et al. 2002), the present investigation was carried out to evaluate the therapeutic efficacy and safety of the antiarthritic compound UC-II (10 mg/day) alone or in a combination with the two most commonly used nutraceutical supplements (glucosamine HCl and chondroitin sulfate) in arthritic dogs given daily for 120 days, followed by a 30-day withdrawal. Glucosamine and chondroitin sulfate have become popular supplements for arthritis and are widely used to ease the pain and discomfort in arthritic dogs. Another objective of this investigation was to determine if the UC-II in combination with glucosamine and chondroitin was well tolerated by arthritic dogs.

MATERIALS AND METHODS

Animals

A group of 20 adult client-owned dogs was selected for this study based on the signs of arthritis, such as joint stiffness, lameness, and pain at the level of moderate severity. These dogs had swollen joints, and were experiencing difficulty in getting up or down (from a sitting and standing position) and walking (horizontal areas and short stairways). Arthritic dogs having any other serious disease or complications (such as hepatic or renal disease) were not included in the study. The owner consent was obtained before initiation of any experiments. The protocol of the present investigation for using arthritic dogs and their treatment was in compliance with the Murray State University Guidelines.

Supplements

UC-II is a standardized, undenatured (native) type II collagen complex containing 10 mg undenatured type II collagen in capsule form for use as a dietary supplement and was provided by InterHealth Nutraceuticals Inc., Benicia, CA, USA. UC-II is manufactured in a GMP facility at a low temperature, which preserves its undenatured form and biological activity. Glucosamine HCl and chondroitin sulfate were also provided as capsules by InterHealth Nutraceuticals.

Experimental

Twenty arthritic dogs were randomly divided into four groups (n=5) and received daily treatment as follows: group I (placebo), group II (10 mg UC-II), group III (2,000 mg glucosamine HCl + 1,600 mg chondroitin sulfate), and group IV (10 mg UC-II + 2,000 mg glucosamine HCl + 1,600 mg chondroitin sulfate). The treatment was given daily for 120 days, followed by a 30-day withdrawal period. None of the dogs received any NSAIDs for 3 to 4 weeks before the study or during the study period. The study was conducted double-blinded; that is, the investigators or owners had no knowledge of the capsule contents.

Pain and Body Weight Measurements

The dogs were evaluated for overall pain, pain upon limb manipulation, and exercise-associated lameness on a monthly basis for a period of 150 days. Overall pain was measured as a general gross observation, which included trouble in standing after sitting, or trouble in sitting after standing, vocalization, crying, etc. Results were graded on a scale of 0 to 10: 0, no pain; 5, moderate pain; and 10, severe and constant pain. Pain upon limb manipulation was evaluated by animals' vocalization or other observations of pain during the extension and flexion of all four limbs for few minutes. Results were graded on a scale of 0 to 4: 0, no pain; 1, mild; 2, moderate; 3, severe; and 4, severe and constant. Lameness was measured after physical exercise for gross observations, which included limping, holding limb up, rigidity of limbs, etc. Signs of pain and lameness were noted on a scale of 0 to 4: 0, no pain; 1, mild; 2, moderate; 3, severe; 4, severe and constant. Severity of pain during various activities, such as standing from sitting, sitting from standing, playing, and vocalization, and during extension and

flexion of limbs was the basis for gradation. Body weights and physical evaluation were determined on a monthly basis. Gross observations were evaluated and recorded monthly using a questionnaire regarding the overall activity and the improvement of the arthritic symptoms. Furthermore, overall performance of individual groups was assessed, which include running, participation in jogging activities, movement up and down stairs, comfort ability in moving from sitting to standing position(s), cheerful attitude toward playing and jumping, etc.

Biochemical Assays

Blood samples were collected by jugular venipuncture using 22-gauge needles and 12-mL syringes. Serum was separated in a marble-top tube (serum separating tubes without anticoagulant) and transferred into plastic snap-top tubes. Serum samples were frozen immediately and kept at −80°C until analyzed for blood urea nitrogen (BUN), creatinine, bilirubin, and alanine aminotransferase (ALT), using Beckman Coulter CX5-PRO Synchron Clinical System (Fullerton, CA). Bilirubin and ALT were used as markers of liver function and BUN and creatinine were used as markers of renal and heart function.

Statistical Analysis

The data of serum chemistry in Table 2 and of pain observation in Figures 1 to 3 are presented as mean ± SEM. Statistical significance of differences was determined by analysis of variance (ANOVA) coupled with Tukey-Kramer test using the NCSS 2000 Statistical System for Windows. Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Data of pain evaluation in arthritic dogs receiving placebo and those receiving UC-II alone or in combination with glucosamine and chondroitin are shown in Figures 1 to 3. Dogs receiving placebo (group I) exhibited no significant change in arthritic conditions at any time during the course of treatment. Dogs receiving UC-II (10 mg/day) alone (group II) showed significant reduction in overall pain within 30 days (33%) and pain upon limb manipulation and exercise-associated lameness after 60 days (66% and 44%, respectively) of treatment. Maximum reductions in the pain were noted after 120 days treatment (overall pain, 62%; pain upon limb manipulation, 91%; and exercise-associated lameness, 78%). Glucosamine plus chondroitin (group III) alleviated some pain but not significantly ($p > 0.05$). Group IV dogs receiving a combination of UC-II and glucosamine plus chondroitin showed marked reductions in overall pain (57%), pain upon limb manipulation (53%), and exercise-associated lameness (53%) after a daily treatment of 120 days. Following the withdrawal of supplements in either of the groups (group II to IV) for 30 days, all dogs experienced a relapse of overall pain, pain upon limb manipulation, and exercise-related lameness. In addition, UC-II-treated dogs were more playful, energetic, and less painful compared to other groups used in this study.

None of the dogs receiving dietary supplements showed any signs of adverse effects. There were no significant changes in any markers of liver function (ALT and bilirubin) or renal or heart function (BUN and creatinine) during the course of this investigation. Body weight remained within the normal range throughout the course of this study. An increase in body weight was observed in the placebo group, demonstrating that activity level in that group was less than the other supplemented group (Table 1).

DISCUSSION

This investigation was pursued with two specific objectives: (a) whether the combination of UC-II and glucosamine plus chondroitin provides better antiarthritic effects than glucosamine plus chondroitin or UC-II alone, and (b) whether these supplements are well tolerated by arthritic dogs, following a long term of their use. The present findings revealed that UC-II therapy (10 mg/day) alone or in combination with glucosamine plus chondroitin for 120 days provided significant improvement in the overall pain, pain upon limb manipulation, and pain after physical exertion. The greatest physical improvements were noted in UC II supplemented group after a treatment period of 120 days, suggesting that prolonged treatment with the supplement may lead to better therapeutic results. After a 30-day withdrawal period, all dogs who had received treatment suffered from a relapse of signs and symptoms associated with arthritic conditions, such as pain and lameness. All supplements were well tolerated and no adverse effects were observed.

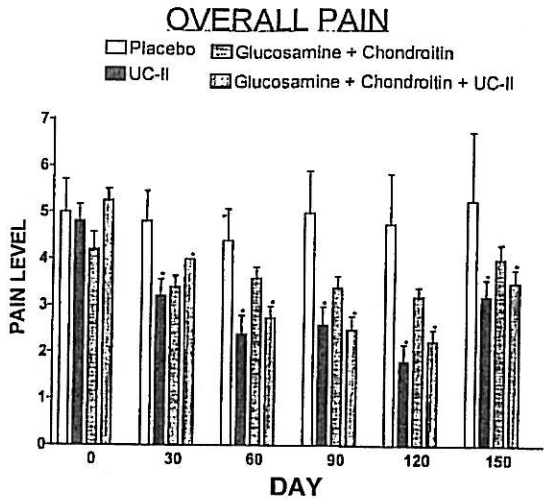

FIGURE 1 Effects of UC-II (10 mg) and/or glucosamine HCl (2,000 mg) plus chondroitin sulfate (1,600 mg) given daily for 120 days, followed by a 30-day withdrawal period, on overall pain in arthritic dogs. Overall pain was graded on a scale of 0 to 10: 0, no pain; 5, moderate pain; and 10, severe and constant pain. * Indicates significant difference from pretreated values ($p < 0.05$).

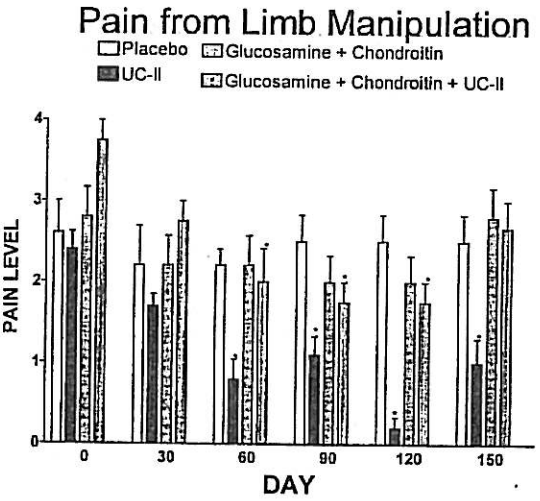

FIGURE 2 Effects of UC-II (10 mg) and/or glucosamine HCl (2,000 mg) plus chondroitin sulfate (1,600 mg) given daily for 120 days, followed by a 30-day withdrawal period, on pain upon limb manipulation in arthritic dogs. Pain upon limb manipulation was evaluated by animal's vocalization or other observations of pain during the extension and flexion of all four limbs for few min. Results were graded on a scale of 0 to 4: 0, no pain; 1, mild; 2, moderate; 3, severe; and 4, severe and constant. * Indicates significant difference from pretreated values ($p < 0.05$).

TABLE 1 Effects of UC-II (10 mg) and/or glucosamine HCl (2,000 mg) plus chondroitin sulfate (1,600 mg) given daily for 120 days, followed by a 30-day withdrawal period, on body weight (pounds) of dogs

Day	Placebo	UC-II	Glucosamine + Chondroitin	Glucosamine + Chondroitin + UC-II
0	69.76 ± 11.49 (100)	62.25 ± 6.29(100)	73.20 ± 7.47 (100)	59.28 ± 9.66 (100)
30	68.88 ± 10.95 (99)	62.35 ± 6.38 (100)	72.80 ± 7.88 (100)	58.83 ± 8.90 (99)
60	69.84 ± 10.86 (100)	62.33 ± 6.55 (100)	73.12 ± 7.31 (100)	57.53 ± 10.04 (96)
90	76.80 ± 10.20 (110)	62.32 ± 6.97 (100)	72.96 ± 7.39 (100)	58.70 ± 9.18 (99)
120	79.58 ± 12.65 (114)	62.83 ± 6.71 (101)	76.16 ± 8.04 (104)	59.53 ± 8.74 (100)

Values are means ± SEM (n = 4–5).
Note: Body weight values remained significantly indifferent throughout the study period compared to pretreated values ($p > 0.05$).

In a recent double-blind pilot study, UC-II (1 mg or 10 mg/day, PO for 90 days) was found to be significantly effective in ameliorating arthritic pain in dogs (DeParle et al. 2005). A 10-mg dose of UC-II provided markedly greater effects in all parameters measured for the arthritic symptoms than 1 mg dose in improving the overall performance and well-being of the dog. The findings of the present study revealed that the arthritic dogs receiving UC-II (10 mg/day) in combination with glucosamine and chondroitin (group IV) overall performed better than glucosamine (2,000 mg) plus chondroitin (1,600 mg) (group III). The most commonly used two nutraceuticals (glucosamine and chondroitin) provided some beneficial antiarthritic effects, but not significantly ($p > 0.05$), which suggested that the observed therapeutic effects were mainly due to the supplementation of UC-II. The findings also revealed that though the beneficial effects of UC-II were observed within 30 days, maximum effects were seen after 120 days. UC-II functions through a process called oral tolerization (i.e., this process takes place in the small intestine, where food is absorbed). Through a complex series of immunological events, patches of lymphoid tissue (Peyer's patches) surrounding the small

TABLE 2 Effects of UC-II (10 mg) and/or glucosamine HCl (2,000 mg) plus chondroitin sulfate (1,600 mg) given daily for 120 days, followed by a 30-day withdrawal period, on markers of liver and renal functions in serum of dogs

	DAY					
	0	30	60	90	120	150
ALT						
Group I	26.00 ± 3.33	22.20 ± 1.98	22.00 ± 1.97	23.75 ± 2.20	22.50 ± 1.66	23.50 ± 1.55
Group II	29.00 ± 2.57	29.00 ± 2.41	30.20 ± 4.08	28.80 ± 3.99	25.20 ± 2.78	25.00 ± 2.19
Group III	21.75 ± 6.97	22.20 ± 3.97	21.00 ± 4.05	19.60 ± 2.75	21.60 ± 2.80	27.20 ± 5.60
Group IV	22.80 ± 3.89	21.50 ± 2.20	23.78 ± 4.82	20.00 ± 4.99	18.25 ± 3.29	23.00 ± 4.82
BILIRUBIN						
Group I	0.52 ± 0.12	0.60 ± 0.16	0.68 ± 0.08	0.43 ± 0.18	0.56 ± 0.09	0.52 ± 0.13
Group II	0.46 ± 0.11	0.52 ± 0.17	0.50 ± 0.08	0.60 ± 0.13	0.70 ± 0.17	0.42 ± 0.13
Group III	0.46 ± 0.12	0.62 ± 0.21	0.40 ± 0.07	0.62 ± 0.14	0.42 ± 0.07	0.50 ± 0.15
Group IV	0.56 ± 0.14	0.48 ± 0.06	0.48 ± 0.03	0.75 ± 0.19	0.50 ± 0.11	0.49 ± 0.06
BUN						
Group I	16.80 ± 1.24	18.00 ± 2.07	13.20 ± 1.65	21.50 ± 1.66	19.00 ± 3.44	17.50 ± 1.32
Group II	17.80 ± 2.65	18.80 ± 2.85	18.40 ± 0.51	18.40 ± 4.72	18.80 ± 1.96	14.40 ± 1.43
Group III	15.20 ± 1.83	16.80 ± 2.10	19.20 ± 2.40	19.60 ± 3.91	15.60 ± 2.04	17.00 ± 1.87
Group IV	19.40 ± 2.58	24.00 ± 2.81	15.75 ± 1.70	21.25 ± 2.29	18.50 ± 3.52	16.00 ± 2.64
CREATININE						
Group I	0.94 ± 0.06	0.92 ± 0.05	0.90 ± 0.03	0.88 ± 0.05	0.87 ± 0.05	0.92 ± 0.05
Group II	1.06 ± 0.07	0.96 ± 0.08	0.98 ± 0.04	0.90 ± 0.08	0.94 ± 0.10	0.96 ± 0.07
Group III	0.92 ± 0.07	0.96 ± 0.05	1.00 ± 0.05	0.94 ± 0.10	0.92 ± 0.07	0.90 ± 0.04
Group IV	0.86 ± 0.02	1.12 ± 0.25	0.80 ± 0.07	0.95 ± 0.07	0.90 ± 0.07	0.87 ± 0.06

Values are means ±SEM (n = 5).
Note: Values of markers of liver function (ALT and bilirubin) and renal function (BUN and creatinine) remained significantly indifferent throughout the study period compared to pretreated values ($p > 0.05$)

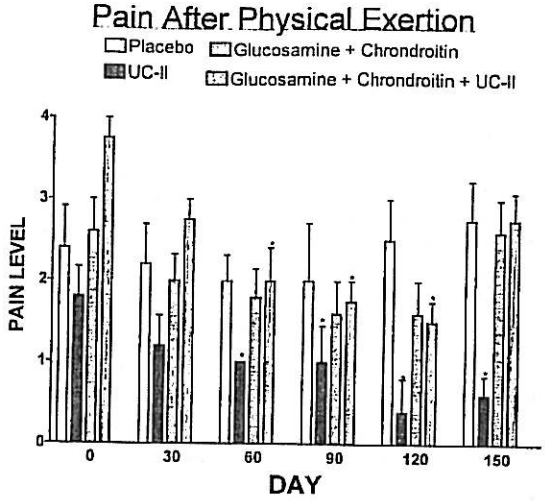

FIGURE 3 Effects of UC-II (10 mg) and/or glucosamine HCl (2,000 mg) plus chondroitin sulfate (1,600 mg) given daily for 120 days, followed by a 30-day withdrawal period, on pain after physical exercise in arthritic dogs. Lameness was measured after physical exercise for limping, holding limb up, rigidity of limbs, etc. Signs of pain and lameness were graded on the scale of 0 to 4: 0, no pain; 1, mild; 2, moderate; 3, severe; and 4, severe and constant. * Indicates significant difference from pretreated values ($p < 0.05$).

intestine screen incoming compounds and serve as a "switch" to turn the body's immune response to foreign substances on or off, depending on what the substance is (Mowat 1987). In the case of type II collagen, small amounts of UC-II (typically 10 mg or less/day, PO) have been shown to turn off the immune response targeted at type II collagen present in bone joint cartilage without any side effects (Weiner et al. 1994; Sieper et al. 1996; Gimsa et al. 1997). This process helps the body to differentiate between elements that are foreign invaders to the body and those that are nutrients (Weiner 1997; Trentham 1998). Previous studies have demonstrated that UC-II improves joint mobility and flexibility by the mechanism of preventing the immune system from attacking and damaging its own joint cartilage (Mowat 1987; Sieper et al. 1996; Gimsa et al. 1997; Trentham 1998; Trentham et al. 1993, 2001).

Unlike UC-II, glucosamine and chondroitin are expected to cause decrease of pain sensation, provide an improved resistance to additional joint tissue breakdown, and rejuvenate some joint tissues. Studies suggest that glucosamine helps to relieve pain by enhancing proteoglycan synthesis, which is impaired in osteoarthritic cartilage (Hougee et al. 2006). Chondroitin sulfate aids in keeping cartilage tissue from dehydrating and assists in cushioning impact stress and reduce joint pain. Earlier studies demonstrated that combining chondroitin sulfate with glucosamine may improve arthritic symptoms and may have some beneficial effects. However, these supplements are not known to reverse structural changes in a joint such as torn cartilage, calcium deposits, and advanced scar tissue. In spite of these beneficial effects of glucosamine and chondroitin described elsewhere, in the present study we did not find significant antiarthritic effects in dogs using the above combination. A recent multicenter with 1,583 patients with symptomatic osteoarthritis in the knee received 1,500 mg of glucosamine and 1,200 mg of chondroitin sulfate daily. They reported that the combination or supplements alone did not reduce pain effectively in the overall treatment groups (Clegg et al. 2006).

Another benefit of the present study resulted in healthy weight maintenance in supplemented groups, compared to control. Body weight was maintained in all supplemented groups, while there was an increase in body weight in the placebo group. This demonstrated that the dogs were more active and arthritic symptoms were remarkably reduced in the supplemented group.

Weight control has been shown to indirectly influence the degenerative joint disease process by reducing the stress on the joint (Eaton 2004). A small amount of weight loss reduces the risk of developing arthritis in dogs and human.

In conclusion, the present study suggests that daily treatment of arthritic dogs with UC-II alone or in combination with glucosamine plus chondroitin ameliorates signs and symptoms of arthritis significantly greater than glucosamine and chondroitin. The results of this study also demonstrate that the supplements are well tolerated. The relapse seen in dogs 30 days posttreatment showed that continuous treatment is required.

REFERENCES

Aceves-Avila, F.J., Medina, F., and Fraga, A. 2001. The antiquity of rheumatoid arthritis: a reappraisal. *J. Rheumatol.* 28:751–757.

Bagchi, D., Misner, B., Bagchi, M., Kothari, S.C., Downs, B.W., and Preuss, H.G. 2002. Effects of orally administered undenatured type II collagen against arthritic inflammatory diseases: a mechanistic exploration. *Intl. J. Clin. Pharmacol. Res.* 22:101–110.

Bellamy, N., Carr, A., Dougados, M., Shea, B., and Wells, G. 2001. Towards a definition of "differences" in osteoarthritis. *J. Rheumatol.* 28:427–430.

Bongartz, T., Sutton, A.J., Sweeting, M.J., Buchan, I., Matteson, E.L., and Montori, V. 2006. Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. *JAMA.* 295:2275–2285.

Center for Disease Control and Prevention. 1994. Prevalence of disability and associated with health conditions-United States, 1991–1992. *MWRW.* 4341:737–259.

Clegg, D.O., Reda, D.J., Harris, C.L., Klein, M.A., O'Dell, J., Hooper, M.M., Bradley, J.D., Bingham, C.O. III., Weisman, M.H., Jackson, C.G., Lane, N.E., Cush, J.J., Moreland, L.W., Schumacher, H.R., Oddis, C.V., Wolfe, F., Molitor, J.A., Yocum, D.E., Schnitzer, T.J., Furst, D.E., Sawitzke, A.D., Shi, H., Brandt, K.D., Moskowitz, R.W., and Williams, H.J. 2006. Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. *N. Engl. J. Med.* 354:795–808.

DeParle, L.A., Gupta, R.C., Canerdy, T.D., Goad, J.T., D'Altilio, M., Bagchi, M., and Bagchi, D. 2005. Efficacy and safety of glycosylated undenatured type-II collagen (UC-II) in therapy of arthritic dogs. *J. Vet. Pharmacol. Ther.* 28:385–390.

Eaton, C.B. 2004. Obesity as a risk factor for osteoarthritis: mechanical versus metabolic. *Med. Health R I.* 87:201–204.

Gimsa, U., Sieper, J., Braun, J., and Mitchison, N.A. 1997. Type II collagen serology: a guide to clinical responsiveness to oral tolerance?. *Rheumatol. Int.* 16:237–240.

Goldring, M.B. 2000. Osteoarthritis and cartilage: the role of cytokines. *Curr. Rheumatol. Rep.* 2:459–465.

Helmick, C.G., Lawrence, R.C., Pollard, R.A., Lloyed, E., and Hayes, S.P. 1995. Arthritis and other rheumatoid conditions: who is affected now, will be affected later? National Arthritic Data Workgroup. *Arthritic Care Res.* 8:203–211.

Hielm-Bjorkman, A.K., Kuusela, E., Liman, A., Markkola, A., Saartr, E., Huffuren, P., Leppaluoto, J., Tulano, R.M., and Raekallio, M. 2003. Evaluation of methods for assessment of pain associated with osteoarthritis in dogs. *J. Am. Vet. Med. Assoc.* 222:1552–1558.

Hougee, S., Hartog, A., Sanders, A., Graus, Y.M., Hoijer, M.A., Garssen, J., Van Den Berg, W., van Beuningen, H.M., and Smit, H.F. 2006. Oral administration of the NADPH-oxidase inhibitor apocynin partially restores diminished cartilage proteoglycan synthesis and reduces inflammation in mice. *Eur. J. Pharmacol.* 531:264–269.

Matteson, E.L. 2000. Historical perspective on the classification of vasculitis. *Arthritis Care Res.* 13:122–127.

Mowat, A.M. 1987. The regulation of immune responses to dietary protein antigens. *Immunol. Today.* 8:93–98.

Muhlfeld, A., and Floege, J. 2005. COX-2 inhibitor induced anuric renal failure in a previously healthy young woman. *Clin. Nephrol.* 63:221–224.

Okada, Y. 2000. Pathoetiology of chronic rheumatoid arthritis-from inflammation to bone destruction. From viewpoint of pathology. *Nppon Naika Gakkai Zassi.* 89:2072–2080.

Paroli, E., Antonilli, L., and Biffoni, M. 1991. A pharmacological approach to glycosaminoglycans. *Drugs Exptl. Clin.* 18:335–343.

Peat, G., McCarney, R., and Croft, P. 2001. Knee pain and osteoarthritis in older adults: a review of community burden and current use of primary health care. *Ann. Rheum. Dis.* 60:91–97.

Physicians Desk Reference. 1998. 52 nd ed. Medical Economics Company Inc., Montvale, NJ, USA.

Richardson, D.C., Schoenherr, W.D., and Zicker, S.C. 1997. Nutritional management of osteoarthritis. *Vet. Clin. North Am. Small Anim. Pract.* 27:883–911.

Sieper, J., Kary, S., Sorensen, H., Alten, R., Eggens, U., Huge, W., Hiepe, F., Kuhne, A., Listing, J., Ulbrich, N., Braun, J., Zink, A., and Mitchison, N. 1996. Oral type II collagen treatment in early rheumatoid arthritis. A double-blind, placebo-controlled, randomized trial. *Arthritis Rheumatol.* 39:41–51.

Trentham, D.E. 1998. Oral tolerization as a treatment of rheumatoid arthritis. *Rheum. Dis. Clin. North Am.* 24:525–536.

Trentham, D.E., Dynesius-Trentham, R.A., Orav, E.J., Bagchi, M., Kothari, S., Pruess, H.G., and Bagchi, D. 1993. Effects of oral administration of type-II collagen on rheumatoid arthritis. *Science* 261:1727–1730.

Trentham, D.E., Halpner, A.D., Trentham, R.A., Combitche, D., Lorenzo, C., Sewell, K.L., Haflir, D.A., and Weiner, H.L. 2001. Use of undenatured type-II collagen in the treatment of rheumatoid arthritis. *Clin. Pract. Alter. Med.* 2:254–259.

Weiner, H.L. 1997. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol. Today.* 18:335–343.

Weiner, H.L., Friedman, A., Miller, A., Khoury, S.J., Al-Sabbagh, A., Santosh, L., Sayegh, M., Nussenblatt, R.B., Trentham, D.E., and Hafler, D.A. 1994. Oral tolerance: immunological mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Ann. Rev. Immunol.* 12:809–837.

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PAIN REDUCTION MEASURED BY GROUND FORCE PLATE IN ARTHRITIC DOGS TREATED WITH TYPE-II COLLAGEN.

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Presently, one in four of 77 million pet dogs in the United States is diagnosed with some form of arthritis. In dogs, osteoarthritis is more common than rheumatoid arthritis and pain is the number one complaint. This investigation evaluated therapeutic efficacy and safety of glycosylated undenatured type II collagen (UC-II) in moderately arthritic dogs that received daily placebo or 40 mg type II collagen (10 mg active UC-II) for a period of 120 days, followed by a 30 day withdrawal. On a monthly basis, dogs were evaluated for overall pain, pain upon limb manipulation, and pain after physical exertion. In addition, pain was measured using Ground Force Plate (peak force and impulse area). Dogs on placebo exhibited no significant change in arthritic conditions. Following 120 days treatment with UC-II, dogs showed significant decreases in overall pain (77%) and pain after limb manipulation (83%) and exercise (84%). With Ground Force Plate, peak vertical force value elevated from 7.467 ± 0.419 to 8.818 ± 0.290 Newtons/kg body wt, and impulse area elevated from 1.154 ± 0.098 to 1.670 ± 0.278 Newtons Sec/kg body wt, suggesting increase in g-force and decrease in level of pain. Dogs receiving placebo or UC-II showed no adverse effects in liver, kidney and heart functions (bilirubin, ALT, creatinine, BUN and CK), or changes in body weight, heart rate, respiration rate, or temperature. In conclusion, UC-II significantly reduced arthritic pain and is well tolerated.

The undenatured type II collagen (UC-II®) used in this study was supplied by InterHealth Nutraceuticals, Benicia, CA.

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Therapeutic efficacy of undenatured type-II collagen (UC-II) in comparison to glucosamine and chondroitin in arthritic horses[1]

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The present investigation evaluated arthritic pain in horses receiving daily placebo, undenatured type II collagen (UC-II) at 320, 480, or 640 mg (providing 80, 120, and 160 mg active UC-II, respectively), and glucosamine and chondroitin (5.4 and 1.8 g, respectively, bid for the first month, and thereafter once daily) for 150 days. Horses were evaluated for overall pain, pain upon limb manipulation, physical examination, and liver and kidney functions. Evaluation of overall pain was based upon a consistent observation of all subjects during a walk and a trot in the same pattern on the same surface. Pain upon limb manipulation was conducted after the walk and trot. It consisted of placing the affected joint in severe flexion for a period of 60 sec. The limb was then placed to the ground and the animal trotted off. The response to the flexion test was then noted with the first couple of strides the animal took. Flexion test was consistent with determining clinically the degree of osteoarthritis in a joint. Horses receiving placebo showed no change in arthritic condition, while those receiving 320 or 480 or 640 mg UC-II exhibited significant reduction in arthritic pain ($P < 0.05$). UC-II at 480 or 640 mg dose provided equal effects, and therefore, 480 mg dose was considered optimal. With this dose, reduction in overall pain was from 5.7 ± 0.42 (100%) to 0.7 ± 0.42 (12%); and in pain upon limb manipulation from 2.35 ± 0.37 (100%) to 0.52 ± 0.18 (22%). Although glucosamine and chondroitin treated group showed significant ($P < 0.05$) reduction in pain compared with pretreated values, the efficacy was less compared with that observed with UC-II. In fact, UC-II at 480 or 640 mg dose was found to be more effective than glucosamine and chondroitin in arthritic horses. Clinical condition (body weight, body temperature, respiration rate, and pulse rate), and liver (bilirubin, GGT, and ALP) and kidney (BUN and creatinine) functions remained unchanged, suggesting that these supplements were well tolerated.

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INTRODUCTION

Arthritis is a chronic debilitating disease that commonly inflicts millions of horses around the world, because of excessive running and exercise, injury, immune disorder, aging, or genetic predisposition (Ruggeiro, 2002). The two most common types of arthritis are osteoarthritis and rheumatoid arthritis. In horses, osteoarthritis occurs with a greater frequency than rheumatoid

arthritis or any other form of joint disease, like in humans and dogs. Osteoarthritis is an inflammatory joint disease, which is characterized by degeneration of the cartilage, hypertrophy of bone at the margins, and changes in the synovial membrane and fluid, which eventually leads to pain and stiffness of joints (Goldring, 2000; Bellamy *et al.*, 2001). This disease can wear down cartilage in a joint to the point that bone rubs against bone, resulting in loss of cartilage, and, in severe cases, cartilage fragments can break off and irritate muscles with pain that are adjacent to the bone. Chronic joint inflammation usually results in progressive joint destruction, deformity, and loss of function (van Roon *et al.*, 2001).

Current therapy of arthritis relies upon nonsteroidal anti-inflammatory drugs (NSAIDs) alone or in combination with some other pain killers. Present treatments aim at alleviating pain, control inflammation, and preserve ability to perform daily functions. NSAIDs, which are cyclooxygenase (COX) inhibitors, alleviate pain, but do not eliminate signs and symptoms of active disease. In general, COX-II inhibitors (such as rofecoxib, celecoxib, carprofen, and deracoxib) are considered safer than nonspecific COX inhibitors (such as aspirin and ibuprofen). In the recent past, chronic use of COX-II inhibitors has been attributed to various side effects, including gastrointestinal (GI) ulceration and bleeding, and hepatic, renal and cardiovascular complications (Richardson, 1991; PDR, 2006; Infante & Lahita, 2000; Matteson, 2000; Schuna & Megeff, 2000; Matheson & Figgilt, 2001; Lamarque, 2004; Solomon *et al.*, 2004; Muhlfeld & Floege, 2005). To our knowledge, such side effects have not been reported in horses.

Presently, nutraceuticals are also used to ease the pain and discomfort of arthritis in both humans and animals, including horses (Trumble, 2005; Clegg *et al.*, 2006; Bruyere & Reginster, 2007; Morva, 2007). These products are commonly used in horses because they are administered orally, well tolerated and considered safe. Nutraceuticals are defined as functional foods, natural products, or parts of food that provide medicinal, therapeutic, or health benefits, including the prevention or treatment of disease. The present investigation utilized three supplements (UC-II, glucosamine, and chondroitin), and their brief description is provided here. Glycosylated undenatured type-II collagen (UC-II) is derived from chicken sternum and prepared under good manufacturing practices (GMPs), using low temperature, which preserves its undenatured form and ensures intact biological activity with active epitopes. Glucosamine, extracted from crab, lobster, or shrimp shells, is an amino-monosaccharide precursor of the disaccharide unit of glycosaminoglycan, which is the building block of proteoglycans, the ground substance of cartilage (Paroli *et al.*, 1991). Chondroitin sulfate, extracted from animal cartilage, such as tracheas and shark cartilage, is a part of a large protein molecule (proteoglycan) that gives cartilage elasticity.

Currently, glucosamine and chondroitin are the two most commonly used nutraceuticals in humans as well as in animals (including dogs, cats, and horses), to alleviate pain associated with arthritis (Dechant *et al.*, 2005; Trumble, 2005). However, based on recent randomized controlled trials and meta-analysis, these supplements have shown only small-to-moderate symptomatic efficacy in human osteoarthritis (Bruyere & Reginster, 2007), although, this finding is still debated (Clegg *et al.*, 2006; Rozendaal *et al.*, 2008). In our recent studies conducted in dogs, daily administration of UC-II at 40 mg (providing 10 mg active UC-II, respectively) daily dose for 120 days markedly reduced arthritic pain (DeParle *et al.*, 2005; D'Altilio *et al.*, 2007). Furthermore, our follow up studies also demonstrated that UC-II (40 mg daily dose) in combination with other nutraceuticals (glucosamine plus chondroitin) markedly reduced the signs associated with arthritis in dogs, and thereby, tremendously improved daily activity, as climbing stairs and walking exercise. In a number of *in vivo* and *in vitro* investigations, glucosamine and chondroitin have been found very effective against osteoarthritis in horses (Fenton *et al.*, 2000, 2002; Dechant *et al.*, 2005; Neil *et al.*, 2005; Trumble, 2005). In brief, these studies suggested that the combination of glucosamine and chondroitin appears to be more effective in preventing or treating osteoarthritis in horses than either product alone.

The present investigation was therefore undertaken with two specific objectives: (i) to determine if daily administration of active UC-II, or glucosamine plus chondroitin, can alleviate the signs and symptoms of arthritis in horses and (ii) to determine if these supplements are well tolerated and safe to administer for the long term in arthritic horses.

MATERIALS AND METHODS

Animals

All horses used in this investigation were diagnosed with osteoarthritis at the level of moderate severity. They were placed at the equine center of Murray State University. During the entire course of investigation, these horses were under the supervision of licensed veterinarians. The protocol of the present investigation for using arthritic horses and their treatment was in compliance with the Murray State University Animal Use and Care Guidelines. All animals were used routinely in their daily workout schedule (riding classes). They were lodged into the amount of time for daily workouts and rest periods. All animals had the same workout protocol and rest time.

Criteria for inclusion into the study

From a large pool of horses located at the Murray State University Equine Center, candidates were chosen based upon outward visual signs of lameness. Once the lame candidates were identified, the animals with evidence of osteoarthritis based upon physical examination by two licensed veterinarians (Dr. Terry D. Canerdy and Dr. William DeWees) were included in the study. Evidence of osteoarthritis includes joint effusion in one or more joints of the limbs, reduced joint flexibility, crepitation of the joint on manipulation, and an increase in lameness upon flexion of the affected joint.

Supplements

Glycosylated undenatured type-II collagen (UC-II), in the form of capsules as a dietary supplement, was provided by InterHealth Nutraceuticals, Inc. (Benicia, CA, USA). Similar to our previous studies conducted in dogs, in the present investigation, the undenatured form of glycosylated type-II collagen was used, as this form of UC-II is found to be significantly more effective than denatured type-II collagen against arthritis (Nagler-Anderson *et al.*, 1986; Bagchi *et al.*, 2002). It should be noted that undenatured type-II collagen can be denatured (hydrolyzed) by chemical or high-temperature, altering its molecular structure and integrity, and denatured collagen does not have active epitopes rendering it inactive. Cosequin equine powder concentrate (glucosamine and chondroitin) was purchased from Nutramax (Edgewood, MD, USA).

Experimental design and animal treatment

The present investigation was conducted on moderately osteoarthritic horses. In preliminary dose-dependent studies, horses received UC-II at 80 or 160 mg (providing 20 and 40 mg active UC-II, respectively) daily dose for a period of 150 days. Based on this pilot dose-dependent study, the final investigation was carried out on five groups of horses (n = 5–6) receiving placebo, UC-II (higher doses), or glucosamine in combination with chondroitin daily for 150 days. Group-I horses received placebo. Horses in Group-II, -III, and -IV received UC-II at 320, 480, and 640 mg (providing 80, 120, and 160 mg active UC-II, respectively), accordingly. Group-V horses received glucosamine and chondroitin (5.4 and 1.8 g/day, respectively, bid for the first month, and once daily thereafter). Treatment in all five groups was given daily (in the form of capsules administered orally in a handful of grain) for a period of 5 months. While rationale for selection of doses of UC-II was based on preliminary studies, doses of glucosamine and chondroitin were based on the product information provided on the insert along with Cosequin (Nutramax).

Pain assessment

The horses were evaluated for overall pain and pain after limb manipulation, on a monthly basis for a period of 150 days. Overall pain evaluation was based upon a consistent observation of all subjects when the animal was at a walk and a trot. All subjects were moved in the same pattern on the same surface consistently. Gross pain measurement was done and recorded during the horses movement trials.

Pain upon limb manipulation was conducted after the walk and trot. It consisted of placing the affected joint in severe flexion for a period of 60 sec. The limb was then placed to the ground and the animal trotted off. The response to the flexion test was then noted with the first couple of strides the animal took. Flexion test was consistent with determining clinically the degree of osteoarthritis in a joint. With an increase in osteophytes, the animal has a degree of discomfort on movement of the limb following flexion. Flexion tests are commonly used in the equine industry in determining the severity of a joint abnormality.

Scale used in pain measurement

The 0–10 global pain assessment was a scale used because it provided a broad range of scale for pain. This scale was consistently used throughout the investigation. In brief, 0, no pain; 5, moderate pain; and 10, severe and constant pain. To our knowledge, a universal scale does not exist to assess the pain.

For pain upon limb manipulation, results were graded on a scale of 0–4: 0, no pain, 1, mild pain; 2, moderate pain; 3, severe pain; and 4, severe and constant pain. The 0–4 scale was taken from the American Association of Equine Practitioners (AAEP) scorecard on lameness. They actually have 0–5, but category 5 was dropped because it indicates inability of an animal to move. None of our subjects fit this category and therefore it was not used.

Physical examination

Body weights and physical evaluation were also determined on a monthly basis for 150 days. On a monthly basis horses were evaluated for body weight, body temperature, and pulse rate.

Biochemical assays

Blood samples were collected by jugular venipuncture using 20-gauge needles and 12-cc syringes. Serum was separated in a marble top tube (without anticoagulant) and transferred into plastic snap-top tubes. Serum samples were frozen immediately and kept at −80 °C until analyzed for bilirubin, GGT, ALP, blood urea nitrogen (BUN) and creatinine, using Beckman Coulter CX5-PRO Synchron Clinical System (Fullerton, CA, USA). Bilirubin, GGT, and ALP were used as markers of liver function, and BUN and creatinine were used as markers of renal function.

Statistical analysis

The data of body weight in Table 1, serum chemistry in Table 2, and pain measurement in Figs 1 & 2, are presented as means ± SEM. Statistical significance of differences was determined by ANOVA coupled with Tukey–Kramer test using the NCSS 2000 Statistical Software for Windows (Kaysville, UT, USA). Groups were compared using Duncan's Multiple-Comparison Test. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Horses used in this investigation were diagnosed with osteoarthritis at a moderate severity. They exhibited some of the common symptoms, such as difficulty during walking, stiffness after periods of inactivity, swelling/tenderness in one or more joints, steady pain in joints, and lameness.

Table 1. Effect of UC-II or Glucosamine plus Chondroitin on body weight (lbs) of horses

Day	Group-I placebo	Group-II 320 mg UC-II	Group-III 480 mg UC-II	Group-IV 640 mg UC-II	Group-V Gluc. + Chon.
0	1161 ± 40 (100)	1080 ± 18 (100)	1150 ± 59 (100)	1190 ± 48 (100)	1195 ± 30 (100)
30	1172 ± 16 (101)	1070 ± 23 (99)	1147 ± 58 (100)	1200 ± 45 (101)	1204 ± 27 (101)
60	1151 ± 48 (99)	1066 ± 22 (99)	1127 ± 53 (98)	1164 ± 49 (98)	1178 ± 33 (99)
90	1131 ± 43 (98)	1068 ± 21 (99)	1137 ± 56 (99)	1178 ± 42 (99)	1185 ± 34 (99)
120	1053 ± 28 (90)	1069 ± 17 (99)	1150 ± 66 (100)	1203 ± 47 (101)	1190 ± 31 (100)
150	1080 ± 39 (93)	1082 ± 23 (100)	1102 ± 59 (96)	1167 ± 33 (98)	1195 ± 49 (100)

Values are means ± SEM (n = 5–7). No significant change in body weight ($P > 0.05$). Numbers in parentheses are percent changes compared with values of day 0 (100%).

Table 2. Effects of UC-II or glucosamine plus chondroitin on markers of liver and renal functions in serum of horses

		Days					
Parameters	Group	0	30	60	90	120	150
BIL (mg/dL)	I	1.18 ± 0.12	1.24 ± 0.12	1.22 ± 0.17	1.34 ± 0.19	1.22 ± 0.16	1.34 ± 0.11
	II	1.33 ± 0.10	1.60 ± 0.06	1.70 ± 0.04	1.30 ± 0.09	1.30 ± 0.03	1.20 ± 0.10
	III	0.98 ± 0.13	1.05 ± 0.13	1.05 ± 0.09	1.15 ± 0.19	1.63 ± 0.27	1.17 ± 0.14
	IV	0.93 ± 0.14	0.77 ± 0.12	1.05 ± 0.12	1.04 ± 0.12	1.30 ± 0.16	0.93 ± 0.10
	V	1.87 ± 0.35	1.84 ± 0.43	1.76 ± 0.27	1.87 ± 0.37	2.07 ± 0.36	2.22 ± 0.48
GGT (IU/L)	I	12.4 ± 2.01	11.4 ± 1.21	11.2 ± 1.68	11.2 ± 1.39	11.8 ± 1.59	13.4 ± 1.21
	II	17.5 ± 9.51	15.4 ± 5.42	14.8 ± 4.82	15.3 ± 8.46	15.1 ± 11.32	14.5 ± 7.90
	III	14.2 ± 1.99	16.2 ± 1.90	13.0 ± 1.51	11.5 ± 1.06	12.7 ± 1.28	12.8 ± 1.35
	IV	14.8 ± 0.79	16.0 ± 0.52	13.1 ± 0.17	13.0 ± 0.58	14.5 ± 0.99	16.6 ± 1.50
	V	12.0 ± 0.69	11.7 ± 0.70	11.5 ± 1.31	12.1 ± 0.73	13.1 ± 0.37	12.1 ± 0.65
ALP (IU/L)	I	95.2 ± 9.61	90.2 ± 7.09	86.4 ± 7.42	94.2 ± 10.18	97.8 ± 14.65	95.4 ± 10.17
	II	79.4 ± 17.91	58.1 ± 22.97	81.3 ± 15.53	87.8 ± 19.38	84.3 ± 22.66	84.5 ± 30.80
	III	84.3 ± 8.50	73.2 ± 5.77	76.7 ± 5.71	74.7 ± 9.43	85.7 ± 12.46	88.3 ± 9.96
	IV	81.5 ± 3.33	68.5 ± 2.84	75.5 ± 3.23	72.8 ± 3.97	77.8 ± 3.66	97.5 ± 4.61
	V	82.6 ± 7.65	77.7 ± 3.98	62.6 ± 6.10	71.4 ± 4.50	75.2 ± 5.50	66.5 ± 7.70
BUN (mg/dL)	I	16.4 ± 0.87	13.6 ± 0.50	14.0 ± 0.89	16.4 ± 1.21	15.2 ± 1.43	18.0 ± 1.34
	II	17.7 ± 1.19	17.9 ± 1.12	17.3 ± 1.08	15.3 ± 1.19	15.1 ± 1.62	14.8 ± 2.00
	III	17.3 ± 1.50	17.1 ± 0.83	16.0 ± 0.86	15.8 ± 0.70	16.3 ± 1.23	18.8 ± 1.14
	IV	18.7 ± 0.88	14.2 ± 0.87	17.3 ± 0.91	19.0 ± 1.13	18.5 ± 0.43	18.8 ± 1.49
	V	18.5 ± 0.50	19.3 ± 0.92	18.9 ± 0.99	16.6 ± 1.74	18.0 ± 1.46	17.2 ± 1.32
Creatinine (mg/dL)	I	1.64 ± 0.08	1.58 ± 0.19	1.66 ± 0.08	1.46 ± 0.12	1.44 ± 0.14	1.66 ± 0.15
	II	1.50 ± 0.07	1.56 ± 0.07	1.51 ± 0.05	1.47 ± 0.07	1.50 ± 0.07	1.35 ± 0.07
	III	1.42 ± 0.06	1.43 ± 0.06	1.58 ± 0.12	1.33 ± 0.07	1.45 ± 0.04	1.48 ± 0.16
	IV	1.52 ± 0.06	1.48 ± 0.06	1.48 ± 0.05	1.30 ± 0.05	1.33 ± 0.02	1.30 ± 0.08
	V	1.43 ± 0.18	1.64 ± 0.19	1.39 ± 0.18	1.53 ± 0.17	1.50 ± 0.15	1.60 ± 0.23

Values are means ± SEM (n = 5–7). No significant change in any parameter ($P > 0.0.5$).

All horses were grossly and physically examined and flexed for lameness on a monthly basis for a period of 150 days. UC-II at a 320, 480, or 640 mg daily dose (providing 80, 120, or 160 mg active UC-II, respectively) provided significant reductions in arthritic pain by 60 days of treatment (Figs 1 & 2). In fact, with higher daily dose of UC-II (480 or 640 mg), significant reduction in overall pain was observed as early as after 30 days of treatment. With UC-II (320 or 480 or 640 mg), horses showed maximal pain reduction by 150 days of treatment (overall pain reduction, 79%, 88%, and 91%, respectively; and pain after limb manipulation, 71%, 78%, and 80%, respectively). After 5 months of UC-II treatment, the horses became very active, and performed normally in their daily activities.

Horses receiving glucosamine (5.4 g) plus chondroitin (1.8 g), bid for the first 30 days, and once daily, thereafter for the next 120 days showed significant decrease in pain after 60 days of treatment (reduction in overall pain, 36%; and reduction in pain after limb manipulation, 31%). Maximal pain reduction was noted after 150 days of treatment (overall pain, 68%; and pain after limb manipulation, 69%). On comparison, the UC-II (480 or 640 mg daily dose) was found to be approximately twice as effective as glucosamine plus chondroitin, based on pain after limb manipulation on day 90.

None of the horses in any group showed any adverse effects on body weight (Table 1), hepatic (bilirubin, GGT, and ALP) or renal (BUN and creatinine) function markers (Table 2), or body temperature, pulse rate, and respiration rate (data not shown), suggesting that these supplements are well tolerated by arthritic horses and safe to administer for a long term.

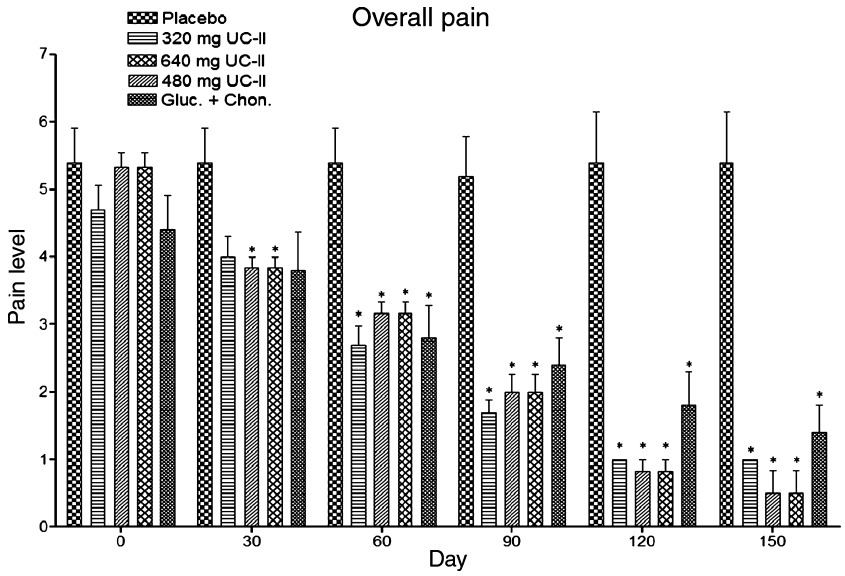

Fig. 1. On a monthly basis, overall pain in horses was measured as a general gross observation and graded on a scale of 0–10: 0, no pain; 5, moderate pain; and 10, severe and constant pain. Values are mean ± SEM (n = 5–7). * = Indicates significant difference between the values of day 0 and post-treatment ($P < 0.05$).

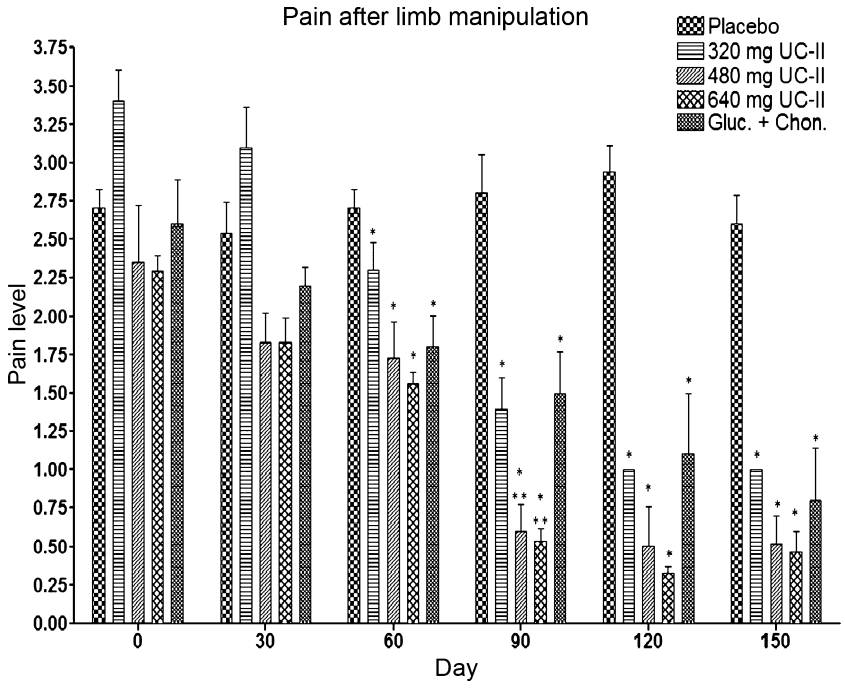

Fig. 2. On a monthly basis, pain upon limb manipulation was evaluated by animal's pain during the flexion of all four limbs for a min. then jogged after each leg was flexed. Results were graded on a scale of 0–4: 0, no pain; 1, mild pain; 2, moderate pain; 3, severe pain; and 4, severe and constant pain. Values are mean ± SEM (n = 5–7). *Significant difference between the values of day 0 and post-treatment ($P < 0.05$). **Significant difference between the values of UC-II-treated and glucosamine plus chondroitin-treated horses ($P < 0.05$).

DISCUSSION

The present investigation evaluated therapeutic efficacy, tolerability, and safety of glycosylated undenatured type II collagen (UC-II) and glucosamine and chondroitin in moderately arthritic horses, following a long term of their use. The present findings revealed that the therapy with UC-II at 320 or 480 or 640 mg daily dose for a period of 5 months provided significant improvement in ameliorating the overall pain and pain after limb manipulation in arthritic horses. Although significant anti-arthritic effects were noted after 60–90 days, the maximal physical improvements were observed after 150 days of treatment and the horses were more playful and active (Figs 1 & 2). This suggests that prolonged treatment with these supplements leads to better therapeutic results. Based on this study, it appears that 480 mg daily dose of UC-II provides the best results, as at further higher dose (640 mg providing 160 mg active UC-II), UC-II offered therapeutic efficacy no greater than that observed with 480 mg daily dose.

Like previous studies conducted in two monogastric species, humans (Nagler-Anderson *et al.*, 1986; Trentham *et al.*, 1993, 2001; Barnett *et al.*, 1996, 1998; Sieper *et al.*, 1996; Trentham, 1998) and dogs (DeParle *et al.*, 2005; D'Altilio *et al.*, 2007), in the horses, we used the undenatured form of UC-II. This form of collagen with triple helix structure and active epitopes is found to be significantly more effective than denatured form against

arthritis (Nagler-Anderson *et al.*, 1986; Bagchi *et al.*, 2002). In none of the species has UC-II been found to produce any adverse effects (Bagchi *et al.*, 2002; D'Altilio *et al.*, 2007), which demonstrated that once UC-II is ingested, stomach acids and enzymes perform a partial digestion of the collagen matrix, resulting in chains of soluble collagen molecules of varying length, containing biologically active epitopes. These structurally precise natural epitopes in UC-II interact with Peyer's Patches and trigger the complex series of immunological events that, in case of rheumatoid arthritis, down-regulates the body's out-of-control autoimmune response (Fig. 3) (Trentham *et al.*, 2001; Bagchi *et al.*, 2002). In the case of osteoarthritis, which is often characterized by a subclinical immune disorder and a vicious cycle of inflammatory events, UC-II can promote a significant reduction in inflammation (Bagchi *et al.*, 2002). UC-II functions through a process of oral tolerization that takes place in the small intestine where the food is absorbed. Through a complex series of immunological events, patches of lymphoid tissue (Peyer's Patches) surrounding the small intestine, screen incoming compounds and serve as a 'switch' to turn the body's immune response to foreign substances on or off, depending upon the substance. In dogs and humans, a small amount of undenatured UC-II (10 mg active UC-II/day) taken orally has been shown to turn off the immune response targeted at type-II collagen in joint cartilage, and no adverse effects have been noted (Trentham *et al.*, 1993, 2001; Trentham, 1998; DeParle *et al.*, 2005). This immunization process helps the body to differentiate between elements that are foreign invaders to the body and those that are nutrients and are good for the body (Weiner, 1997; Trentham, 1998). UC-II stops the immune system from attacking and damaging its own joint cartilage, thereby improving joint mobility and flexibility (Trentham *et al.*, 1993; Trentham, 1998; Bagchi *et al.*, 2002). Type-II collagen is one of the primary connective tissues of the body, providing flexibility and support to bone joints. As UC-II is found to be as equally effective in horses, as reported earlier in humans and dogs, and it is presumed that the mechanisms described for humans and dogs may also hold true for horses. Although the precise biochemical mechanism involved in UC-II- induced pharmacological anti-arthritic effects in humans, dogs or horses, is not clearly established.

Glucosamine and chondroitin (5.4 and 1.8 g, respectively, bid for the first 30 days, and once daily for the next 120 days) significantly reduced arthritic pain by 60 days of treatment (Figs 2 & 3), but maximal pain reduction was observed after 150 days (68% in overall pain and 69% in pain after limb manipulation). Recently, a number of *in vivo* and *in vitro* studies support the use of glucosamine and chondroitin in arthritic horses (Fenton *et al.*, 2000, 2002; Dechant *et al.*, 2005; Neil *et al.*, 2005; Trumble, 2005). Unlike UC-II, glucosamine relieves pain by enhancing proteoglycan synthesis, which is impaired in osteoarthritic cartilage (Hougee *et al.*, 2006). Chondroitin sulfate aids in keeping cartilage tissue from dehydrating and assists in cushioning impact stress and reducing joint pain. Chondroitin sulfate is also believed to block certain enzymes that result in the breakdown of cartilage. In an *in vitro* study, Dechant *et al.* (2005) demonstrated that glucosamine plus chondroitin: (i) reduced total glycosaminoglycan degradation, which is involved in osteoarthritis and (ii) have no detrimental effects on cartilage metabolism. Furthermore, from a series of *in vitro* studies, Fenton *et al.* (2000, 2002) revealed that glucosamine can prevent experimentally induced cartilage degradation, and therefore support the use of this product in prevention or treatment of cartilage loss in arthritic horses. In a recent *in vivo* study, glucosamine and chondroitin ameliorated arthritic pain in dogs, but comparatively UC-II was significantly more effective. Similarly, in horses UC-II (480 or 640 mg daily dose) was found to be more effective compared with glucosamine and chondroitin based upon limb manipulation on 90 days of treatment.

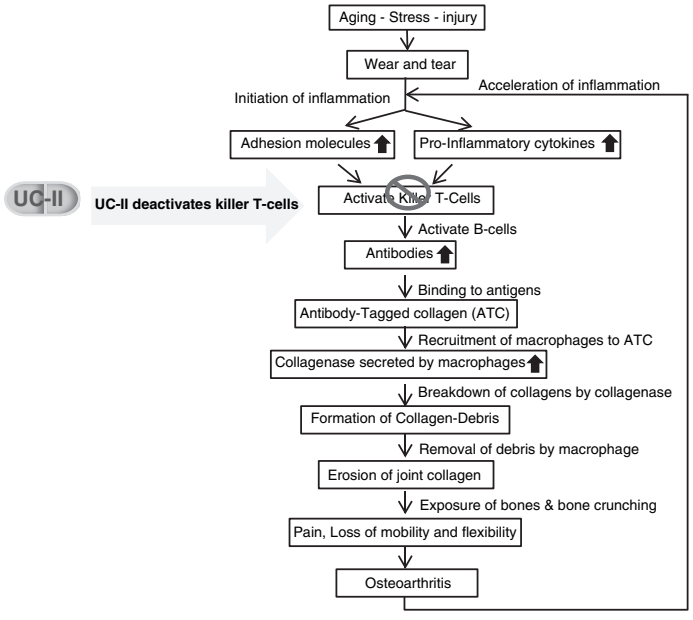

Fig. 3. Mechanism of action of UC-II in osteoarthritis.

In conclusion, daily administration of UC-II at varying doses (320 or 460 or 640 mg) significantly reduced the signs and symptoms of arthritis in horses. Daily administration of glucosamine plus chondroitin also provided reduction in arthritic pain, but the efficacy was less than UC-II. All three supplements were well tolerated and did not produce any adverse events.

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REFERENCES

Bagchi, D., Misner, B., Bagchi, M., Kothari, S.C., Downs, B.W., Fafard, R.D. & Preuss, H.G. (2002) Effects of orally administered undenatured type II collagen against arthritic inflammatory diseases: a mechanistic exploration. *International Journal of Clinical Pharmacology Research*, **22,** 101–110.

Barnett, M.L., Combitchi, D. & Trentham, D.E. (1996) A pilot trial of oral type II collagen in the treatment of juvenile rheumatoid arthritis. *Arthritis and Rheumatism*, **39,** 623–628.

Barnett, M.L., Kremer, J.M., St. Clair, E.W., Clegg, D.O., Furst, D., Weisman, M., Fletcher, M.J.F., Chasan-Taber, S., Finger, E., Morales, A., Le, C.H. & Trentham, D.E. (1998) Treatment of rheumatoid arthritis with oral type II collagen: results of a multi-center, double-blind, placebo-controlled trial. *Arthritis and Rheumatism*, **41,** 290–297.

Bellamy, N., Carr, A., Dougados, M., Shea, B. & Wells, G. (2001) Towards a definition of ''differences'' in osteoarthritis. *Journal of Rheumatology*, **28,** 427–430.

Bruyere, O. & Reginster, J.Y. (2007) Glucosamine and chondroitin sulfate as therapeutic agents for knee and hip osteoarthritis. *Drugs and Aging*, **24,** 573–580.

Clegg, D.O., Reda, D.J., Harris, C.L., Klein, M.A., O'Dell, J.R., Hooper, M.M., Bradley, J.D., Bingham, C.O. III, Weisman, M.H., Jackson, C.G., Lane, N.E., Cush, J.J., Moreland, L.W., Schumacher, H.R. Jr, Oddis, C.V., Wolfe, F., Molitor, J.A., Yocum, D.E., Schnitzer, T.J., Furst, D.E., Sawitzke, A.D., Shi, H., Brandt, K.D., Moskowitz, R.W. & Williams, H.J. (2006) Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. *New England Journal of Medicine*, **354** (8), 795–808.

D'Altilio, M., Peal, A., Alvey, M., Simms, C., Curtsinger, A., Gupta, R.C., Canerdy, T.D., Goad, J.T., Bagchi, M. & Bagchi, D. (2007) Therapeutic efficacy and safety of undenatured type II collagen singly or in combination with glucosamine and chondroitin in arthritic dogs. *Toxicology Mechanisms and Methods*, **17,** 189–196.

Dechant, J.E., Baxter, G.M., Frisble, D.D., Trotter, G.W. & McIlwraith, C.W. (2005) Effects of glucosamine hydrochloride and chondroitin sulfate, alone and in combination, on normal and interleukin-1 conditioned equine articular cartilage explants metabolism. *Equine Veterinary Journal*, **37,** 227–231.

DeParle, L.A., Gupta, R.C., Canerdy, T.D., Goad, J.T., D'Altilio, M., Bagchi, M. & Bagchi, D. (2005) Efficacy and safety of glycosylated undenatured type II collagen (UC-II) in therapy of arthritic dogs. *Journal of Veterinary Pharmacology and Therapeutics*, **28,** 385–390.

Fenton, J.I., Chlebek-Brown, K.A., Peters, T.L., Caron, J.P. & Orth, M.W. (2000) Glucosamine HCl reduces equine articular cartilage degradation in explants culture. *Osteoarthritis and Cartilage*, **8,** 258–265.

Fenton, J.I., Chlebek-Brown, K.A., Caron, J.P. & Orth, M.W. (2002) Effect of glucosamine on interleukin-1-conditioned articular cartilage. *Equine Veterinary Journal. Supplement*, **34,** 219–223.

Goldring, M.B. (2000) Osteoarthritis and cartilage: the role of cytokines. *Current Rheumatology Reports*, **2,** 459–465.

Hougee, S., Hartog, A., Sanders, A., Graus, Y.M., Hoijer, M.A., Garssen, J., Van Den Berg, W., Van Beuningen, H.M. & Smit, H.F. (2006) Oral administration of the NADPH-oxidase inhibitor apocynin partially restores diminished cartilage proteoglycan synthesis and reduces inflammation in mice. *European Journal of Pharmacology*, **531,** 264–269.

Infante, R. & Lahita, R.G. (2000) Rheumatoid arthritis: new disease-modifying and anti-inflammatory drugs. *Geriatrics*, **55,** 30–32.

Lamarque, D. (2004) Safety of selective inhibitors of inducible cyclo-oxygenase-2 take a long period. *Bulletin of Cancer*, **91,** S117–S124.

Matheson, A.J. & Figgilt, D.P. (2001) Rofecoxib: a review of its use in the management of osteoarthritis, acute pain and rheumatoid arthritis. *Drugs*, **61,** 833–865.

Matteson, E.L. (2000) Current treatment strategies for rheumatoid arthritis. *Mayo Clinic Proceedings*, **75,** 69–74.

Morva, T. (2007) Glucosamine studies on horses. http: EzineArticles.com/?expert. pp. 1–3.

Muhlfeld, A.S. & Floege, J. (2005) COX-2 inhibitor induced anuric renal failure in a previously healthy young woman. *Clinical Nephrology*, **63,** 221–224.

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. *Proceedings of National Academy of Science (USA)*, **83,** 7443–7446.

Neil, K.M., Orth, M.W., Coussens, P.M., Chan, P.S. & Caron, J.P. (2005) Effects of glucosamine and chondroitin sulfate on mediators of osteoarthritis in cultured equine chondrocytes stimulated by use of recombinant equine interleukin-1beta. *American Journal of Veterinary Research*, **66,** 1861–1869.

Paroli, E., Antonilli, L. & Biffoni, M. (1991) A pharmacological approach to glycosaminoglycans. *Drugs Under Experimental and Clinical Research*, **18,** 335–343.

Physicians' Desk Reference (PDR) (2006), 60nd edn. Thomson, Montavale, NJ.

Richardson, D.W. (1991) Treatment of degenerative joint disease. *Veterinary Review*, **11,** 210–212.

van Roon, J.A., Lafeber, F.P. & Bijlsma, J.W. (2001) Synergistic activities of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction of rheumatoid arthritis. *Arthritis and Rheumatism*, **44,** 3–12.

Rozendaal, R.M., Koes, B.W., van Osch, G.V., Uitterlinden, E.J., Garling, E.H., Willemsen, S.P., Zinai, A.Z., Verhaar, J.A., Weinans, H. & Bierma-Zeinstra, S.M. (2008) Effect of glucosamine sulfate on hip osteoarthritis. *Annals of Internal Medicine*, **148,** 268–277.

Ruggeiro, B. (2002) Arthritis in horses. Essortment. October 22, 2005. http://me.essortment.com/arthritisinho_rfgt.htm.

Schuna, A.A. & Megeff, C. (2000) New drugs for the treatment of rheumatoid arthritis. *American Journal of Health and Systematic Pharmacology*, **57,** 225–234.

Sieper, J., Kary, S., Sorenson, H., Alten, R., Effens, U., Huge, W., Hiepe, F., Kuhne, A., Listing, J., Ulbrich, N., Braun, J., Zink, A. & Mitchison, N. (1996) Oral type II collagen treatment in early rheumatoid arthritis: a double-blind, placebo-controlled, randomized trial. *Arthritis Rheumatology*, **39,** 41–51.

Solomon, D.H., Schneeweiss, S., Glynn, R.J., Kiota, Y., Levin, R. & Mogun, H.A.J. (2004) Relationship between selective cyclooxygenase-2 inhibitors and myocardial infarction in older adults. *Circulation*, **109,** 2068–2073.

Trentham, D.E. (1998) Oral tolerization as a treatment of rheumatoid arthritis. *Rheumatic Diseases Clinics of North America*, **24,** 525–536.

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

Trentham, D.E., Halpner, A.D., Trentham, R.A., Bagchi, M., Kothari, S., Preuss, H.G. & Bagchi, D. (2001) Use of undenatured type II collagen in the treatment of rheumatoid arthritis. *Clinical Practice of Alternative Medicine*, **2,** 254–259.

Trumble, T.N. (2005) The use of nutraceuticals for osteoarthritis in horses. *Veterinary Clinics of North America. Equine Practice*, **21,** 575–597.

Weiner, H.L. (1997) Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunology Today*, **18,** 335–343.

J. vet. Pharmacol. Therap. **30**, 275–278, doi: 10.1111/j.1365-2885.2007.00844.x. SHORT COMMUNICATION

Therapeutic efficacy and safety of undenatured type-II collagen (UC-II) alone or in combination with (−)-hydroxycitric acid and chromemate in arthritic dogs[1]

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The present investigation evaluated therapeutic efficacy and safety of glycosylated undenatured type II collagen (active UC-II) alone or in combination with (−)-hydroxycitric acid (HCA-SX, SuperCitrimax) and ChromeMate (chromium niacinate, CM). Twenty five arthritic dogs in five groups ($n = 5$) received daily treatment as follows: group I (placebo), group II (10 mg active UC-II), group III (1800 mg HCA-SX), group IV (1800 mg HCA-SX + 100 μg CM), and group V (1800 mg HCA-SX + 100 μg CM + 10 mg active UC-II). The treatment was given daily for 120 days, followed by 30 days withdrawal. The dogs were evaluated for overall pain, pain upon limb manipulation, and exercise-associated lameness, on a monthly basis. Blood-serum samples were assayed for markers of liver [bilirubin and alanine aminotrasferase (ALT)] and renal [blood urea nitrogen (BUN) and creatinine] functions. Group I dogs exhibited no significant change in arthritic conditions. The dogs receiving active UC-II alone (group II) or in combination with HCA-SX + CM (group V) for 90 days showed marked reduction in overall pain (46–57%), pain upon limb manipulation (50–55%), and exercise-associated lameness (44–46%). In groups II and V, maximum pain reduction was seen after 120 days treatment (62–70%, 67–91%, and 69–78%, correspondingly). All dogs experienced a relapse of pain after a withdrawal period of 30 days. None of the dogs in any group showed adverse effects or changes in liver or kidney function markers, or body temperature. Body weights of all dogs remained significantly unchanged in all the groups. These data suggest that treatment of arthritic dogs with active UC-II alone or in combination with HCA-SX and CM ameliorates the signs of arthritis, and these supplements are well tolerated as no adverse effects were noted.

Arthritis is a chronic degenerative disease of the joints causing pain, stiffness, swelling, and lameness (McLaughlin, 2000; Burns, 2006). Arthritis commonly affects large breed dogs (Richardson *et al.*, 1997), because of overweight/obesity, lack of exercise, physical injury, aging, infection, immune disorder, or genetic predisposition. Dogs suffer more often with osteoarthritis than with rheumatoid arthritis (Hielm-Bjorkman *et al.*, 2003). Osteoarthritis is an inflammatory joint disease, which is characterized by degeneration of the cartilage, hypertrophy of bone at the margins in the synovial membrane, and eventually pain and stiffness of joints (Vaughan-Scott & Taylor, 1997).

Present therapy for arthritis in dogs relies upon drugs that alleviate pain and control inflammation to preserve daily activity. Chronic use of cyclooxygenase (COX) inhibitors (nonsteroidal anti-inflammatory drugs, NSAIDs) is linked to numerous side effects, including gastrointestinal (GI) bleeding, and hepatic and renal dysfunction (Lobetti & Joubert, 2000; Bergh & Budsberg, 2005). In the recent past, two commonly used FDA-approved drugs (Rimadyl and Deramaxx), which are NSAIDs and selective inhibitors of COX-II, have been shown to cause severe side effects (Moreau *et al.*, 2003; Sessions *et al.*, 2005).

In recent years, InterHealth Nutraceuticals, Inc. (Benicia, CA, USA) has developed three supplements (active UC-II, SuperCitrimax, and ChromeMate) that are proven to be very effective in human arthritis and/or obesity patients (Bagchi *et al.*, 2002;

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Soni *et al.*, 2004; Shara *et al.*, 2005). The structural integrity of undenatured type II collagen in a active UC-II sample was determined by Transmission Electron Microscope procedure using an EM JEOL 100 CX (Peabody, MA, USA), while the amount of undenatured type-II was analyzed by Capture ELISA kit (Chondrex LLC, Redmond, WA, USA) (Bagchi *et al.*, 2002).

Twenty-five client-owned arthritic dogs weighing between 62 and 96 pounds were used in this investigation. These dogs exhibited the signs of osteoarthritis (joint stiffness, lameness, pain, swollen joints, and difficulty in getting up or down and walking), which was confirmed radiographically. Dogs were randomly divided into five groups ($n = 5$) receiving daily treatment as follows: group I (placebo), group II (10 mg active UC-II), group III (1800 mg HCA-SX), group IV (1800 mg HCA-SX + 100 μg CM); and group V (1800 mg HCA-SX + 100 μg CM + 10 mg active UC-II). Daily treatment was given for 120 days, followed by a 30-day withdrawal.

Overall pain, pain upon limb manipulation, and lameness after physical exertion was measured on a monthly basis for a period of 150 days. Grading for pain measurement is described in figure legends (Figs 1–3), and in our recent publications (DeParle *et al.*, 2005; D'Altilio *et al.*, 2007).

Data of pain assessment are shown in Figs 1–3. Dogs receiving placebo showed no improvement in arthritic pain or lameness. Dogs receiving active UC-II alone showed significant reduction in overall pain, pain upon limb manipulation, and exercise-associated lameness. Maximum improvement was noted after 120 days of treatment. HCA-SX alone did not provide significant improvement in pain reduction, but in combination with CM, it provided significant reductions in arthritic signs, including pain. Active UC-II in combination with HCA-SX and CM markedly reduced overall pain (70%), pain upon limb manipulation (67%), and exercise-associated lameness (69%). Following a 30-day withdrawal, dogs experienced a relapse of pain and lameness. Data of dogs' body weight, body temperature, and serum chemistry related to liver and renal function (bilirubin, ALT, BUN, and creatinine), did not show any significant changes at 0, 30, 60, 90, 120, and 150 days.

Recently, in a double-blinded pilot study, we found for the first time that active UC-II (1 or 10 mg/day) given for 90 days significantly reduced the pain in arthritic dogs (DeParle *et al.*, 2005). Dogs given 10 mg active UC-II performed overall better than those given a 1-mg dose. In a follow-up study, dogs receiving active UC-II (10 mg/day) alone or in combination with Glucosamine HCl (2000 mg/day) and Chondroitin sulfate (1600 mg/day) for 120 days showed significant reductions in pain (D'Altilio *et al.*, 2007). The present data revealed that daily therapy with active UC-II alone or with HCA-SX + CM for 120 days provided remarkable improvements in the lifestyle of dogs by reducing arthritic pain. The majority of anti-arthritic effects appeared to be obtained from active UC-II, which exerts its effects through a process of oral tolerization (Trentham, 1998; DeParle *et al.*, 2005; D'Altilio *et al.*, 2007). Dogs receiving these supplements were more playful and showed significant reductions in the signalments of the arthritic condition, including pain and lameness (Figs 1–3).

In conclusion, arthritic dogs treated with active UC-II alone or in combination with HCA-SX and CM showed marked reductions in arthritic pain and lameness. Overall, the dogs became very active and playful. The supplements did not

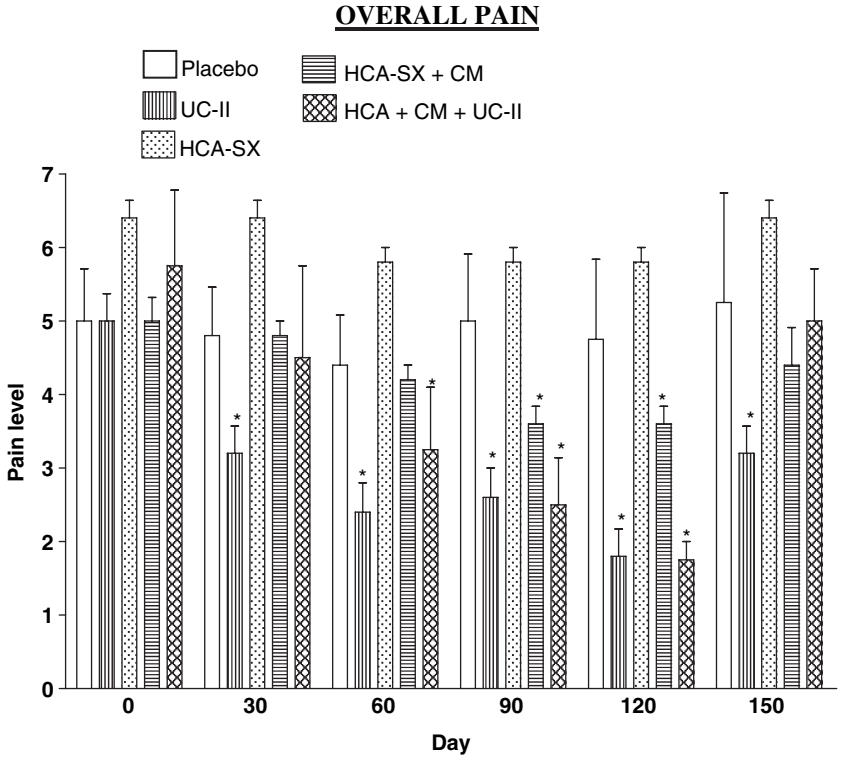

Fig. 1. Effects of active UC-II alone (10 mg/dog/day) or in combination with HCA-SX (1800 mg/dog/day) + CM (100 μg/dog/day) on overall pain in arthritic dogs ($n = 5$ dogs/group). Daily treatment continued for 120 days, followed by a withdrawal period of 30 days. Overall pain was graded on a scale of 0–10: 0, no pain; 5, moderate; and 10, severe and constant pain. For details, see the text and DeParle *et al.* (2005). *Significantly different when compared with pretreated values ($P < 0.05$). Active UC-II, glycosylated undenatured type-II collagen; HCA-SX, (−)-hydroxycitric acid; and CM, ChromeMate.

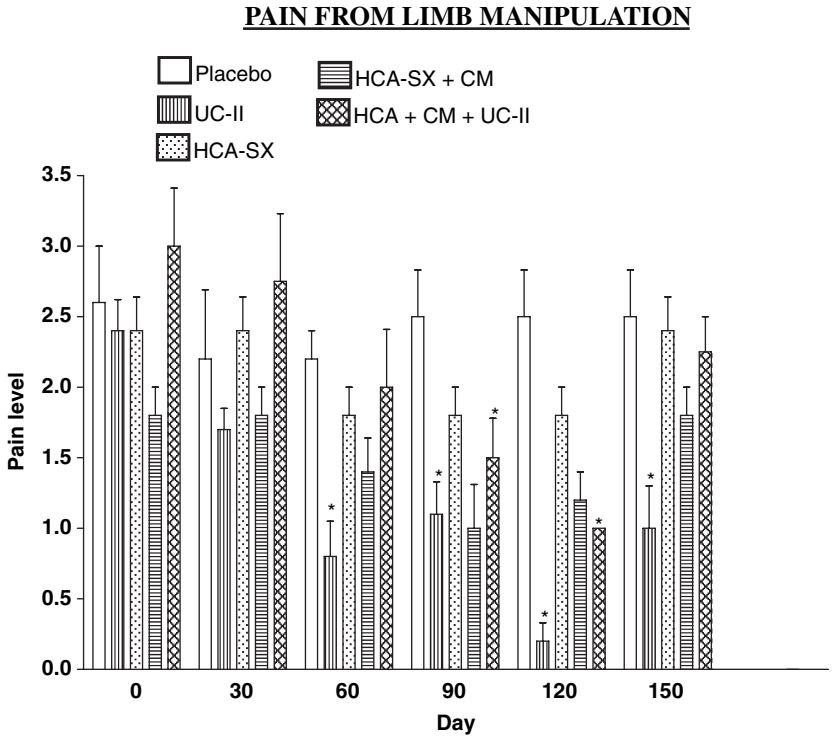

Fig. 2. Effects of active UC-II alone or in combination with HCA-SX + CM on pain after limb manipulation. Pain was evaluated by animal's vocalization or other observations of pain during the extension and flexion of all four limbs for few min. Pain was graded on a scale of 0–4: 0, no pain; 1, mild; 2, moderate; 3, severe; and 4, severe and constant pain. For details, see the text and Fig. 1. *Significantly different when compared with pretreated values ($P < 0.05$).

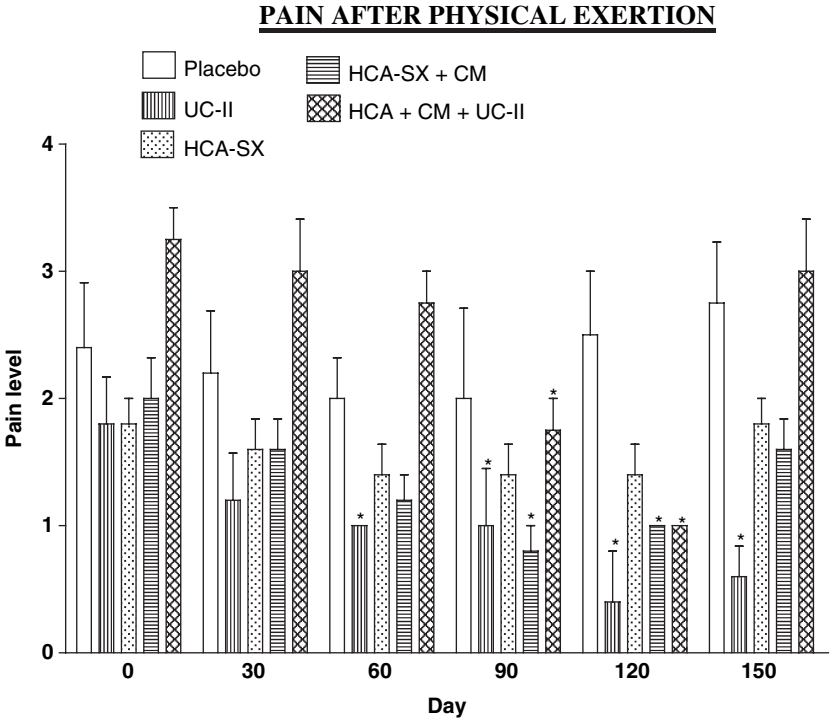

Fig. 3. Effects of active UC-II alone or in combination with HCA-SX + CM on pain after physical exertion. Lameness was measured after physical exercise for limping, holding limb up, rigidity of limbs, etc. Signs of pain and lameness were graded on the scale of 0–4: 0, no pain; 1, mild; 2, moderate; 3, severe; and 4, severe and constant pain. For details, see the text and Fig. 1. *Significantly different when compared with pretreated values ($P < 0.05$).

produce any side effects and were well tolerated. Relapse of arthritic signs, seen following a 30-day withdrawal, suggests that continuous therapy is needed. These data suggest that active UC-II, HCA-SX, and CM are well tolerated and safe to use with great efficacy in arthritic dogs.

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REFERENCES

Bagchi, D., Misner, B., Bagchi, M., Kothari, S.C., Downs, B.W., Fafard, R.D. & Preuss, H.G. (2002) Effects of orally administered undenatured type II collagen against arthritic inflammatory diseases: a mechanistic exploration. *International Journal of Clinical Pharmacology Research*, **22,** 101–110.

Bergh, M.S. & Budsberg, S.C. (2005) The coxib NSAIDs: potential clinical and pharmacologic importance in veterinary medicine. *Journal of Veterinary Internal Medicine*, **19,** 633–643.

Burns, K. (2006) Research targets and conditions of older cats and dogs. *Journal of the American Veterinary Medical Association*, **229,** 482–483.

D'Altilio, M., Peal, A., Curtsinger, A., Alvey, M., Simms, C., Gupta, R.C., Canerdy, T.D., Goad, J.T., Bagchi, M. & Bagchi, D. (2007) Therapeutic efficacy and safety of undenatured type II collagen singly or in combination with glucosamine and chondroitin in arthritic dogs. *Toxicology Mechanisms and Methods*, **17** (in press).

DeParle, L.A., Gupta, R.C., Canerdy, T.D., Goad, J.T., D'Altilio, M., Bagchi, M. & Bagchi, D. (2005) Efficacy and safety of glycosylated undenatured type-II collagen (UC-II) in therapy of arthritic dogs. *Journal of Veterinary Pharmacology and Therapeutics*, **28,** 385–390.

Hielm-Bjorkman, A.K., Kuusela, E., Liman, A., Markkola, A., Saarto, E., Huttunen, P., Leppaluoto, J., Tulamo, R.M. & Raekallio, M. (2003) Evaluation of methods for assessment of pain associated with osteoarthritis in dogs. *Journal of the American Veterinary Medical Association*, **222,** 1552–1558.

Lobetti, R.G. & Joubert, K.E. (2000) Effect of administration of non-steroidal anti-inflammatory drugs before surgery on renal function in clinically normal dogs. *American Journal of Veterinary Research*, **61,** 1501–1507.

McLaughlin, R. (2000) Management of chronic osteoarthritic pain. *Veterinary Clinics of North America. Small Animal Practice*, **30,** 933–949.

Moreau, M., Dupuis, J., Bonneau, N.H. & Desnoyers, M. (2003) Clinical evaluation of a nutraceutical, carprofen and meloxicam for the treatment of dogs with osteoarthritis. *Veterinary Record*, **152,** 323–329.

Richardson, D.C., Schoenherr, W.D. & Zicker, S.C. (1997) Nutritional management of osteoarthritis. *Veterinary Clinics of North America. Small Animal Practice*, **27,** 883–911.

Sessions, J.K., Reynolds, L.R. & Budsberg, S.C. (2005) In vivo effects of carprofen, deracoxib, and etodolac on prostanoid production in blood, gastric mucosa, and synovial fluid in dogs with chronic osteoarthritis. *American Journal of Veterinary Research*, **66,** 812–817.

Shara, M., Yasmin, T., Kincaid, A.E., Limpach, A.L., Bartz, J., Brenneman, K.A., Chatterjee, A., Bagchi, M., Stohs, S.J. & Bagchi, D. (2005) Safety and toxicological evaluation of a novel niacin-bound chromium (III) complex. *Journal of Inorganic Biochemistry*, **99,** 2161–2183.

Soni, M.G., Burdock, G.A., Preuss, H.G., Stohs, S.J., Ohia, S.E. & Bagchi, D. (2004) Safety assessment of (−)-hydroxycitric acid and Super Citrimax, a novel calcium/potassium salt. *Food and Chemical Toxicology*, **42,** 1513–1529.

Trentham, D.E. (1998) Oral tolerization as a treatment of rheumatoid arthritis. *Rheumatic Diseases Clinics of North America*, **24,** 525–536.

Vaughan-Scott, T. & Taylor, J.H. (1997) The pathophysiology and medical management of canine osteoarthritis. *Journal of the South African Veterinary Association*, **68,** 21–25.

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RESEARCH ARTICLE

Safety and toxicological evaluation of undenatured type II collagen

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Abstract

Previous research has shown that undenatured type II collagen is effective in the treatment of arthritis. The present study evaluated the broad-spectrum safety of UC-II by a variety of toxicological assays including acute oral, acute dermal, primary dermal irritation, and primary eye irritation toxicity. In addition, genotoxicity studies such as Ames bacterial reverse mutation assay and mouse lymphoma tests, as well as a dose-dependent 90-day sub-chronic toxicity study were conducted. Safety studies indicated that acute oral LD_{50} of UC-II was greater than 5000 mg/kg in female Sprague-Dawley rats. No changes in body weight or adverse effects were observed following necropsy. Acute dermal LD_{50} of UC-II was determined to be greater than 2000 mg/kg. Primary skin irritation tests conducted on New Zealand Albino rabbits classified UC-II as slightly irritating. Primary eye irritation tests conducted on rabbits indicated that UC-II was moderately irritating to the eye. UC-II did not induce mutagenicity in the bacterial reverse mutation test in five *Salmonella typhimurium* strains either with or without metabolic activation. Similarly, UC-II did not induce a mutagenic effect in the gene mutation test in mouse lymphoma cells either with or without metabolic activation. A dose-dependent 90-day sub-chronic toxicity study revealed no pathologically significant changes in selected organ weights individually or as percentages of body or brain weights. No significant changes were observed in hematology and clinical chemistry. Therefore, the results from the current study show a broad-spectrum safety profile of UC-II.

Keywords: *Undenatured type II collagen; 90-day toxicity study; acute oral toxicity; acute dermal toxicity; primary dermal toxicity; primary eye irritation; body and selected organ weights; hematology and clinical chemistry; histopathology*

Introduction

Arthritis and its related chronic conditions affect one in every five Americans, thus representing one of the most prevalent causes of disability in the US (Helmick et al. 2008). Indeed, over 46 million US adults suffered from doctor-diagnosed arthritis in 2008. This number is estimated to rise to 67 million by 2030, a massive 46% increase, due in part to increases in obesity and longevity (Helmick et al. 2008). There are more than 100 different types of arthritis and among them osteoarthritis (OA) is by far the most prevalent form, affecting ~ 60% of all arthritis sufferers Lawrence et al. 2008). Rheumatoid arthritis (RA) is the second most common form of arthritis, impinging on 1.3 million US adults (Helmick et al. 2008). Arthritis describes chronic conditions characterized by joint pain and difficulty in performing certain tasks resulting in limited activity (Trentham 1984; 1996; Trentham et al. 1993; 2001; Barnett et al. 1996; 1998). Consequently, arthritis imposes a tremendous socioeconomic burden on the US public health system and diminishes the quality of life of millions of people. OA is the second most common chronic disease leading to Social Security disability payments due to long-term absence from work (Bitton 1999). It is prevalent in the aging population and affects roughly 12% of people aged 60 or older (Felson 2009).

OA is defined by the American College of Rheumatology as a heterogeneous group of conditions characterized by degeneration of articular cartilage and changes in the underlying bone at the joint margins (Altman et al. 1986). The etiopathogenesis of OA is multifactorial, and includes inflammatory, metabolic, and mechanical components. A number of risk

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factors such as genetics, dietary intake, muscle weakness, obesity, and trauma may initiate various pathogenic pathways leading to OA (Felson et al. 2000). In spite of considerable medical advances in recent years, there is little effective treatment for OA. Common non-surgical treatments of OA include cyclooxygenase-2 (COX-2) inhibitors and non-steroidal anti-inflammation drugs (NSAIDs) targeting pain and inflammation. Unfortunately, many of these agents show limited efficacy and are associated with serious side-effects and high toxicities (Sarzi-Puttini et al. 2005). These side-effects include renal and upper gastrointestinal adverse events, increased risk for cardiovascular events, and elevated blood pressure (Sarzi-Puttini et al. 2005; Berenbaum 2008). In addition, the recent negative press and the withdrawal of certain COX-2 selective NSAIDs from the market have prompted many OA-sufferers to seek alternative therapies. There is a growing recognition of the important role of nutraceuticals in the maintenance of bone and joint health (Goggs et al. 2005). Among these nutraceuticals, a natural collagen extract known as UC-II has gained considerable attention recently for its demonstrated efficacy in the treatment of OA (Crowley et al. 2009).

UC-II is a undenatured type II collagen derived from chicken sternum cartilage. Animal studies (Deparle et al. 2005; D'Altilio et al. 2007; Peal et al. 2007; Bagchi et al. 2008a; 2009; Gupta et al. 2009a; b) and human trials (Bagchi et al. 2008b; Crowley et al. 2009) have demonstrated UC-II to be effective and safe in treating OA. A quantitative evaluation of the therapeutic efficacy of UC-II for 120 days was assessed in osteoarthritic dogs using a Ground Force Plate (GFP) procedure which objectively measures the peak force and impulse area (Gupta et al. 2009b). Dogs on placebo exhibited no significant change in arthritic conditions. UC-II supplemented dogs exhibited a significant improvement, as indicated by GFP analysis. The peak force was increased by 18% and impulse area was elevated by 44%, suggesting an increase in *g*-force and a decrease in level of pain.

The beneficial effects of UC-II on OA was also observed in horses (Gupta et al. 2009a). Osteoarthritic horses were supplemented with placebo, UC-II (320, 480, or 640 mg) or a combination of 5400 mg of glucosamine plus 1800 mg of chondroitin for 150 days. Horses receiving 320, 480, or 640 mg of UC-II exhibited significant reduction in arthritic pain. UC-II at a dose of 480 or 640 mg provided equal effects, and,therefore, 480 mg was considered optimal. With this dose, there was an 88% decrease in overall pain and a 78% decrease in pain upon limb manipulation. UC-II was found to be more effective in reducing arthritic pain than glucosamine plus chondroitin (Gupta et al. 2009a).

A recent human clinical trial further demonstrated the safety and efficacy of UC-II in the treatment of OA (Crowley et al. 2009). A randomized, double-blind clinical study was conducted in North America on 52 people with OA of the knee. A daily dose of 40 mg of UC-II was more than twice as effective as 1500 mg of glucosamine plus 1200 mg of chondroitin in promoting joint health after 90 days. UC-II significantly decreased joint pain, discomfort, and immobility compared to baseline, and outperformed the glucosamine plus chondroitin combination using three standard OA assessments: Western Ontario and McMaster Osteoarthritis Index (WOMAC), Visual Analog Scale (VAS), and Lequesne Functional Index.

The objective of the present study was to determine the safety profile of UC-II and hence acute oral toxicity, acute dermal toxicity, primary dermal irritation, primary eye irritation, mutagenicity, and 90-day sub-chronic toxicity studies were conducted by in vivo and in vitro procedures.

Materials and methods

Study compound

UC-II is a unique, patented natural collagen concentrate containing 25% undenatured type II collagen. UC-II (UC-250, off white powder) was obtained from InterHealth Research Center (Benicia, CA) and used in all the studies reported here.

Animals and treatment

Safety tests were conducted at Eurofins/Product Safety Laboratories (Dayton, NJ) in compliance with the Good Laboratory Practices (GLP) as defined in 21CFR58 by the US Food and Drug Administration (FDA, 1987) and in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines for testing of chemicals (OECD 1998). The mutagenicity studies were performed at Bioservice Scientific Laboratories (Planegg, Germany) in compliance with GLP as defined in the Chemikaliengesetz (Chemical Act) of the Federal Republic of Germany (BGB1. I Nr. 50 S. 2407), and in accordance with the Environmental Directorate published by OECD in the Series on Principles of Good Laboratory Practice and Compliance Monitoring (OECD 1998). Animals were cared for in accordance with the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Detailed animal protocols are provided in individual toxicological assessments.

Acute oral toxicity

The acute oral toxicity evaluation (Up and Down Procedure) was conducted in rats to determine the potential of UC-II to produce acute oral toxicity from a single dose through the oral route. Six healthy young adult female, nulliparous, and non-pregnant albino Sprague Dawley rats (aged 9–10 weeks old, initial body weight 188–197 g) were obtained from Ace Animals, Inc. (Boyertown, PA). Female rats were selected for the test because they are frequently more sensitive to the toxicity of test compounds than males. The female rats were singly housed in suspended stainless steel cages with mesh floors conforming to the size recommendations in the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Litter paper was placed beneath the cage and was changed at least three times per week. The rats had free access to standard rat chow (Purina Rodent Chow# 5012) and filtered tap water ad libitum, and were maintained at controlled temperature (20–24°C) and light cycle (12 h light/12 h dark). The animals were acclimated to

laboratory conditions at least 10–14 days prior to initiation of dosing.

UC-II was administered in sequence to the animals, as described in Table 1. The decision to proceed with the next animal was based on the survival of the previous animal following dosing. Before each dosing, rats were fasted overnight, examined through the fasting period for health, and weighed (initial). Individual doses were calculated based on initial body weights at a dose level of 5000 mg/kg. UC-II was administered as a 14% w/w suspension in distilled water using a stainless steel ball-tipped gavage needle. Following administration, each animal was returned to its designated cage and the feed was replaced 3–4 h after the final dosing. Individual body weights were recorded again on days 7 and 14 (termination) following dosing. The animals were observed for mortality, signs of gross toxicity, and behavioral changes during the first several hours post-dosing and at least once daily thereafter for 14 days after dosing. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavioral pattern. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhea, and coma. All rats were euthanized by CO_2 inhalation at the end of the 14-day observation period and gross necropsies were performed on all animals. Tissues and organs of the thoracic and abdominal cavities were examined.

Acute dermal toxicity

The acute dermal toxicity evaluation was conducted in rats to determine the potential for UC-II to produce toxicity from a single topical application. Five healthy young adult albino Sprague Dawley male rats (aged 10–11 weeks old, initial body weight 290–307 g) and five young adult female, nulliparous, and non-pregnant albino Sprague Dawley rats (aged 10–11 weeks old, initial body weight 200–215 g) were obtained from Ace Animals, Inc. (Boyertown, PA). The rats were singly housed in suspended stainless steel cages with mesh floors. Litter paper was placed beneath the cage and was changed at least three times per week. The rats had free access to standard rat chow (Purina Rodent Chow# 5012) and filtered tap water ad libitum, and were maintained at controlled temperature (19–23°C) and light cycle (12 h light/12 h dark). The animals were acclimated to laboratory conditions for 21 days.

On the day prior to UC-II application, the five male and five female animals were prepared by clipping (Oster model #A5 -small) the dorsal area and the trunk. After clipping and prior to application, the animals were examined for health, weighed (initial), and the skin checked for any abnormalities. Individual doses were calculated based on the initial body weights, taking into account the concentration of the test mixture. Prior to application, UC-II was moistened with distilled water to achieve a dry paste by preparing a 50% w/w mixture. UC-II (2000 mg/kg of body weight) was then applied to a 2 × 3-inch 4-ply gauze pad and placed on a dose area of ~ 2 × 3 inches (~ 10% of the body surface). The gauze pad and entire trunk of each animal were then wrapped with 3-inch Durapore tape to avoid dislocation of the pad and to minimize loss of UC-II. The rats were then returned to their designated cages. The day of application was considered as day 0 of the study. After 24 h of exposure of UC-II, the pads were removed, and the test sites were gently cleansed of any residual test substance. Individual body weights of the animals were recorded prior to UC-II application (initial) and again on days 7 and 14 (termination). The animals were observed for mortality, signs of gross toxicity, and behavioral changes during the first several hours after application and at least once daily thereafter for 14 days. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavioral pattern. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhea, and coma. All rats were euthanized via CO_2 inhalation on day 14. Gross necropsies were performed on all animals at terminal sacrifice. Tissues and organs of the thoracic and abdominal cavities were examined.

Primary dermal irritation

The primary dermal irritation test was conducted in two young adult male New Zealand albino rabbits and one young nulliparous non-pregnant female New Zealand albino rabbit to determine the potential for UC-II to cause irritation after a single topical application. The rabbits were obtained from Robinson Services, Inc. (Clemmons, NC), and singly housed in suspended stainless steel cages with mesh floors, which conform to the size recommendations in the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Litter paper was placed beneath the cage and was changed at least three times per week. The rabbits were allowed free access to lab chow (Purina Rabbit Chow # 5326, St. Louis, MO) and filtered tap water ad libitum, and maintained at controlled temperature (20–22°C) and light cycle (12 h light/12 h dark). Animals were acclimated to

Table 1. Acute oral toxicity dosing sequence and observations.

Dosing sequence	Dose level (mg/kg)	Body weight (g)			Cage-side observations (days 0–14)	Necropsy observations (all tissues)
		Initial	Day 7	Day 14		
1	175	182	207	243	Active and healthy	No gross abnormalities
2	550	205	224	253	Active and healthy	No gross abnormalities
3	1750	181	200	246	Active and healthy	No gross abnormalities
4	5000	200	220	257	Active and healthy	No gross abnormalities
5	5000	177	198	244	Active and healthy	No gross abnormalities
6	5000	186	200	246	Active and healthy	No gross abnormalities

laboratory conditions for a period of 28 days prior to initiation of dosing.

On the day before application, rabbits were prepared by clipping (Oster model #A5 -small) the dorsal area and the trunk. On the day of dosing but prior to application, the rabbits were critically examined for health and the skin checked for any abnormalities, and three healthy rabbits without pre-existing skin irritation were selected for the test. Individual doses were calculated based on the initial body weights, taking into account the concentration of the test mixture. On the day of application (day 0), UC-II was moistened with distilled water to achieve a dry paste by preparing a 50% w/w mixture. Five-tenths of a gram of UC-II (1.0 g of test mixture) was placed on a 1 × 1-inch 4-ply gauze pad and applied to one 6-cm^2 intact dose site on each rabbit. The pad and entire trunk of each rabbit were then wrapped with semi-occlusive 3-inch Micropore tape to avoid dislocation of the pad. Elizabethan collars were placed on each rabbit and they were returned to their designated cages. After 4 h of exposure to UC-II, the pads and collars were removed and the test sites were gently cleansed of any residual test substance.

Individual dose sites were scored according to the Draize scoring system (Table 2) (Draize et al. 1944) at ~ 1, 24, 48, and 72 h after patch removal. The classification of irritancy was obtained by adding the average erythema and edema scores for the 1, 24, 48, and 72-h scoring intervals and dividing by the number of evaluation intervals (four). The animals were also observed for signs of gross toxicity and behavioral changes at least once daily during the test period. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavior pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhea, and coma.

Primary eye irritation

The primary eye irritation test was conducted in rabbits to determine the potential for UC-II to produce irritation from a single installation through the ocular route. Three female, nulliparous and non-pregnant New Zealand albino rabbits were obtained from Robinson Services, Inc. (Clemmons, NC) and singly housed in suspended stainless steel cages with mesh floors, which conform to the size recommendations in the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Litter paper was placed beneath the cage and was changed at least three times per week. The rabbits were allowed free access to lab chow (Purina Rabbit Chow# 5326, St. Louis, MO) and filtered tap water ad libitum, and maintained at controlled temperature (17–24°C) and light cycle (12 h light/12 h dark). Animals were acclimated to laboratory conditions for a period of 22 days prior to initiation of dosing.

Table 2. Primary dermal irritation index (PII) and classification.

Primary Dermal Irritation Index (PDII)	Classification
0	Non-irritating
> 0–2.0	Slightly irritating
2.1–5.0	Moderately irritating
> 5.0	Severely irritating

Prior to instillation, both eyes of rabbits were examined using a fluorescein dye procedure. One drop of 2% ophthalmic fluorescein sodium was instilled into both eyes of each rabbit. The eyes were rinsed with physiological saline (0.9% NaCl) ~ 30 s after installation of the fluorescein. Using an ultraviolet light source, the eyes were checked for gross abnormalities according to the 'Scale for Scoring Ocular Lesions' (Draize et al. 1944). Three healthy animals without pre-existing ocular irritation were selected for the test. One-tenth of a milliliter (0.06 g) of UC-II was instilled into the conjunctival sac of the right eye of each rabbit by gently pulling the lower lid away from the eyeball. The upper and lower lids were then gently held together for ~ 1 s before releasing to minimize loss of the test substance. The left (control) eye of each animal remained untreated and served as a control. The rabbits were then returned to their designated cages. Ocular irritation was evaluated macroscopically using a high-intensity white light in accordance with Draize et al. (1944) at 1, 24, 48, and 72 h, and 4 days post-instillation. The fluorescein eye evaluation was used at 24 h to verify the absence of corneal damage. Individual irritation scores were recorded for each animal. In addition to observations of the cornea, iris, and conjunctivae, any other lesions were noted. The average score for all rabbits at each scoring period was calculated to aid in data interpretation. Time intervals with the highest mean score (Maximum Mean Total Score; MMTS) for all rabbits were used to classify the test substance (UC-II) by the system of Kay and Calandra (1962).

The animals were also observed for signs of gross toxicity and behavioral changes at least once daily during the test period. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavior pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhea, and coma.

Mutagenicity test: Ames' bacterial reverse mutation assay

The *Salmonella typhimurium* reverse mutation test (Maron and Ames 1983) was conducted to determine the ability of UC-II to induce reverse mutation. UC-II was evaluated in the Ames/Salmonella plate incorporation assay to determine its potential to induce reverse mutation at selected histidine loci in five tester strains of *Salmonella typhimurium* viz. TA 1535, TA 97a, TA 98, TA 100, and TA 102 in the presence and absence of a metabolic activation system (S9) (Ames et al. 1977). Suspensions of bacterial cells were exposed to UC-II in triplicate cultures at concentrations of 10.1, 31.6, 100, 316, 1000, 2500, and 5000 μg/plate in the presence and absence of an exogenous metabolic activation system (S9). The suspensions were mixed with an overlay agar and plated immediately onto minimal medium. After 48 h incubation, revertant colonies were counted using a ProtoCOL counter (Meintrup DWS Laborgerate, GmbH) and compared to the number of spontaneous revertant colonies on vehicle (negative) control plates.

Mutagenicity test: Mouse lymphoma assay

The mutagenic potential of UC-II was evaluated by in vitro mammalian cell gene mutation assay (Thymidine Kinase Locus/$TK^{+/-}$) in mouse (*Mus musculus*) lymphoma cell line L5178Y. The assay was performed in both the presence and absence of an exogenous metabolic activation system at the gene locus coding for the enzyme thymidine kinase (TK) in mouse lymphoma cells.

UC-II was investigated at the following concentrations: Experiment I with and without metabolic activation, 200, 400, 600, 800, 1000, 1200, 1500, and 2000 μg/ml; Experiment II with metabolic activation, 300, 500, 700, 1100, 1400, 1800, and 2000 μg/ml; and Experiment II without activation, 4.4, 17.6, 39.6, 70.4, 110, 264, 330, and 440 μg/ml. The selection of concentrations was based on data from the pre-experiment. In experiment I, 2000 μg/ml (with and without metabolic activation) was selected as the highest concentration. In experiment II, 2000 μg/ml (with metabolic activation) and 440 μg/ml (without metabolic activation) were selected as the highest concentration. Experiment II without metabolic activation was performed as a 24 h long-term exposure assay. Ethylmethanesulfonate (EMS), methylmethanesulfonate (MMS), and benzo[a]pyrene (B[a]P) were used as positive controls. Each trial consisted of duplicate cultures of the negative (vehicle) and positive controls, and single cultures treated at each of the dosage levels of UC-II described above. Treatment consisted of 11 ml of the appropriate treatment medium (with or without exogenous activation), designated concentrations of UC-II and 1×10^7 cells in a $25cm^2$ flask, and incubated at 37°C in 5% CO_2/95% humidified air. After 4 h incubation, the test compound was removed by centrifugation (200 x *g*, 10 min) and the cells were washed twice with phosphate buffered saline (PBS). The cells were suspended in 30 ml complete culture medium and incubated for an expression and growth period of 72 h. For the long-term exposure experiment, 1×10^7 cells were suspended in 50 ml cell culture medium in a 175-cm^2 flask. After expression and growth period, the relative cloning efficiency (RCE; percentage cloning efficiency of the test group in relation to the negative control) of the cells was determined as previously described (Clive and Spector 1975; Clive 1983; Clive et al. 1983; Mitchell et al. 1997).

Dose-dependent 90-day sub-chronic toxicity study

A 90-day oral toxicity study was conducted in male and female rats at Eurofins/Product Safety Laboratories (Dayton, NJ) to determine the potential of UC-II to produce toxicity. A no-observed-adverse-effect level (NOAEL) was also sought for each sex. Eighty healthy rats (40 males and 40 females) were selected for the test and equally distributed into four groups (10 males and 10 females per dose level) according to Table 3.

Animal selection

After acclimating to the laboratory environment for 7 days, the rats were examined for general health and weighed. Only those rats free of clinical signs of disease or injury and having a body weight range within ± 20% of the mean were selected for test. The animals weighed in the range of 195–219 g for males and 148–174 g for females, and were ~ 7–8 weeks of age at test initiation. The 40 male and 40 female rats were randomly distributed, stratified by body weight, among the dose and control groups on the day prior to study start.

Dose preparations

The test substance was administered as a 0.4% (low dose), 4.0% (intermediate dose), or 10.0% (high dose) weight/weight dilution in distilled water. On each dosing day and for each concentration, an appropriate amount of the test substance was accurately weighed into a 150 mL glass beaker and distilled water was added until the desired total weight was obtained. The dose preparations were used at room temperature within ~ 2 h, and maintained on a magnetic stir plate during administration.

Dose calculations

Individual doses were calculated based on the most recent weekly body weights and were adjusted each week to maintain the targeted dose level for all rats. All doses were administered volumetrically after correcting for dilution. Doses were administered to all groups at a constant dose volume of 10.0 mL/kg. The control group received the vehicle only (distilled water) at the same volume as the test animals.

Dose administration

Each animal was dosed by oral intubation to the stomach using a ball-tipped gavage needle attached to an appropriate syringe. Dosing was 7 days per week for a period of 92 days for males and 93 days for females. The first day of administration was considered Day 1 of the study. Dosing was at approximately the same time each day ± 2 h, with an exception on the days the hematology and/or clinical chemistry samples were collected. On the days of blood collection, food was returned to the fasted animals for a minimum of 2 h prior to test substance administration.

Ophthalmologic evaluations

Prior to study initiation, the eyes of a group of rats considered for study were examined by focal illumination and indirect ophthalmoscopy. Mydriasis was achieved with 1% tropicamide and the eyes were examined in subdued light. Subdued light was maintained in the animal room for the remainder of the day. This procedure was repeated on Day 91 for all surviving test animals.

Clinical observations

All animals were observed at least twice daily for viability. Cage-side observations of all animals were performed daily during the study or until death occurred. On Day 1 (prior to first treatment with the test substance) and approximately weekly thereafter, a detailed clinical observation test was conducted while handling the animals, generally on days that the animals were weighed and food consumption measurements taken. Potential signs noted included, but were not

Table 3. Dose levels and assignment of animals.

Group	Number/group	Number/sex	Oral gavage dose (mg/kg/day)	Dose volume (ml/kg/day)	% UC-II
1	20	10	Control (0)	10.0	0
2	20	10	Low dose (40)	10.0	0.4
3	20	10	Intermediate dose (400)	10.0	4.0
4	20	10	High dose (1000)	10.0	10.0

See Materials and methods section for details.

limited to changes in skin, fur, eyes, and mucous membranes, occurrence of secretions, excretions, and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern). Likewise, changes in gait, posture, and response to handling, as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling), or bizarre behavior (e.g. self-mutilation, walking backwards) were also recorded.

Body weight, organ weight, and body weight gain

Individual body weights were recorded twice during the acclimation period, on Day 0 (the day of study start) and approximately weekly thereafter (7 day intervals ± 1). Mean daily body weight gains were calculated for each sex and dose level at each interval and for the overall (Days 1–92) testing interval. Animals were also weighed prior to sacrifice (fasted body weight) for the calculation of organ-to-body weight and organ-to-brain weight ratios. The following organs were weighed wet as soon as possible after dissection to avoid drying: liver, kidneys (combined), adrenals (combined), brain, heart, thymus, spleen, ovaries (combined) or testes (combined), epididymides, and uterus and fallopian tubes.

Food consumption and food efficiency

Individual food consumption was measured and was recorded weekly adjusting for spillage. Mean daily food consumption was calculated for each sex/dose level during each weekly interval and overall (Days 1–92) testing interval. Mean daily food efficiency was also calculated for each sex/dose level based on body weight gain and food consumption data. Animals were allowed ad libitum access to food throughout the study. Animals were fasted overnight prior to blood collection on Day 90, and prior to terminal sacrifice on Day 92 (males) or Day 93 (females).

Functional observational battery

A Functional Observational Battery (FOB) was performed on all animals on Day 86 (females) and Day 87 (surviving males). Each rat was evaluated during handling and while in an open field for excitability, autonomic function, gait and sensorimotor coordination (open field and manipulative evaluations), reactivity and sensitivity (elicited behavior), and other abnormal clinical signs including but not limited to convulsions, tremors, unusual or bizarre behavior, emaciation, dehydration, and general appearance. In addition to the above observations, forelimb and hind limb grip strength and foot splay measurements were obtained and recorded. The grip strength was measured with a digital force gauge (Wagner Force Five, Model #FDMV). Triplicate measurements of grip strength and duplicate measurements for foot splay were taken for each animal and the means for each group were calculated.

Motor activity

Motor Activity (MA) was evaluated on all surviving animals on Day 86 (males) and Day 87 (females). This assessment was done at approximately the same period during the study as the FOB. Activity was monitored using an automated Photobeam Activity System® (San Diego Instruments, Inc.). An approximate equal number of animals from each dose group were assigned to the MA assessment for each session. Each animal was placed into a polycarbonate solid bottom cage, room lights were turned off, and a white noise generator was used. The evaluation phase began immediately for that animal. Each animal was evaluated for a single 1-h phase, with photobeam counts accumulated over six 10-min intervals. Total movements (consisting of fine movements and active movements) were considered an appropriate measure for the assessment of potential behavioral effects in this study.

Clinical pathology

All surviving animals were fasted overnight prior to each blood collection. Blood samples for hematology (except coagulation samples) and clinical chemistry were collected via the sub-lingual vein under isoflurane anesthesia during the 12th week of exposure for males and females. Approximately 500 µl was collected in a pre-calibrated tube containing EDTA for hematology assessments. The whole blood samples were stored under refrigeration and shipped on cold packs. Approximately 1000 µl was collected into tubes containing no preservative for clinical chemistry assessments. These samples were centrifuged in a refrigerated centrifuge and the serum was transferred to a labelled tube. Serum samples were stored in a –80°C freezer and shipped frozen in dry ice. All samples were shipped to DuPont Haskell Global Centers for Health and Environmental Sciences (Newark, DE). Blood samples used to determine the prothrombin time and activated partial thromboplastin time (coagulation) were collected via the inferior vena cava under isoflurane anesthesia at terminal sacrifice. Approximately 1800 µl were collected in a pre-calibrated tube containing sodium citrate. These samples were centrifuged in a refrigerated centrifuge and the plasma was transferred to a labelled tube. Plasma samples were stored in a –80°C freezer and shipped frozen in dry ice to DuPont Haskell Global Centers for Health and Environmental Sciences. The day before collection of the samples for the clinical pathology evaluation, the animals

were placed in metabolism cages. These animals were fasted after 3 pm (at least 15 h) and urine was collected from each animal. Urine samples were stored under refrigeration and shipped on cold packs to DuPont Haskell Global Centers for Health and Environmental Sciences. All blood samples were evaluated for quality by visual examination. Upon completion of clinical chemistry, remaining serum samples from two randomly selected animals were pooled at DuPont Haskell and sent to Charles River Diagnostics (Wilmington, MA) for serology.

Sacrifice and macroscopic observations

Scheduled sacrifice. At terminal sacrifice, all surviving males (Day 93) and all females (Day 94) were euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia. All animals in the study (including decedents) were subjected to a full necropsy, which included examination of the external surface of the body, all orifices, and the thoracic, abdominal, and cranial cavities, and their contents. Additional tissues were preserved if indicated by signs of toxicity or target organ involvement.

Histopathology. Histological examination was performed on the preserved organs and tissues of the animals from both the control and high dose groups (Groups 1 and 4, respectively) as well as from any animal that died during the course of the study. In addition, gross lesions of potential toxicological significance noted in any test groups at the time of terminal sacrifice were also examined. Due to findings in the males and females of Group 4 high dose, the nasal turbinates were evaluated in the intermediate Group 3 animals. The fixed tissues were trimmed, processed, embedded in paraffin, microtomed, placed on glass microscope slides, stained with hematoxylin and eosin, and examined by light microscopy. Slide preparation and histopathological assessment was performed by Histo-Scientific Research Laboratories (Mt. Jackson, VA).

Statistical analysis

Eurofins/Product Safety Laboratories performed statistical analysis of all data collected during the in-life phase of the study as well as organ weight data. DuPont Haskell Laboratory provided analysis of clinical pathology results to Eurofins/Product Safety Laboratories. The use of the word 'significant' or 'significantly' indicates a statistically significant difference between the control and the experimental groups. Significance was judged at a probability value of $p \leq 0.05$. Male and female rats were evaluated separately.

Statistical methods (in-life and organ weight data)

Group means and standard deviations were calculated for body weight, daily body weight gain, daily food consumption, daily food efficiency, organ weight, and organ-to-body/brain weight ratio, FOB and MA data. Data within groups were compared using a One-Way of Analysis (ANOVA), followed by comparison of the treated groups to control by Dunnett's Multiple Comparisons test. Data were evaluated for homogeneity of variances and normality by the Bartlett's test. Data that were considered significant by Bartlett's test were further evaluated with a non-parametric method (Kruskal-Wallis or Dunn's test) (INSTAT Biostatistics, Graph Pad Software, San Diego, CA). Motor activity data (overall total movements) were further analyzed using a Two-Way Repeated Measures ANOVA (SigmaStat, Version 2.03).

Statistical methods (clinical pathology)

Means and standard deviations were calculated for clinical pathology quantitative data. Data within groups were initially analyzed using Levene's test for variance homogeneity, and the Shapiro-Wilk test for normality. If variances were considered not significantly different, groups were compared using a One-Way Analysis of Variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. If the Shapiro-Wilk test was not significant but Levene's test was significant, a robust version of Dunnett's test was used. Where variances were considered significantly different by Levene's test, groups were compared using a non-parametric method (Kruskal-Wallis non-parametric analysis of variance followed by Dunn's test). Differences among groups were judged significant at a probability value of $p \leq 0.05$. Male and female rats were evaluated separately.

Results

Acute oral toxicity

A single oral administration of UC-II was provided to female Sprague-Dawley rats to assess its acute toxicity following Up and Down procedure. UC-II, at the limit dose of level of 5000 mg/kg body weight, did not cause any mortality and did not demonstrate any signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior in the treated female rats following dosing and during the observation period of 14 days thereafter. All animals survived, gained normal body weight, and appeared active and healthy during the study. No gross abnormalities or pathological alterations were noted for any of the rats when necropsied at the conclusion of the 14-day observation period (Table 1). Based on these results and under the conditions of this study, the acute oral LD_{50} of UC-II is greater than 5000 mg/kg of body weight in female rats.

Acute dermal toxicity

Acute dermal toxicity of UC-II was conducted in male and female Sprague Dawley rats to determine the potential for UC-II to cause toxicity from a single topical application. All animals survived, gained normal body weight, and appeared active and healthy during the study. There were no signs of dermal irritation, gross toxicity, adverse pharmacologic effects, or abnormal behavior. No gross abnormalities were noted for any of the animals when necropsied at the conclusion of the 14-day observation period. The findings are summarized in Table 4. Under the conditions of this study, the single dose acute dermal LD_{50} of UC-II is greater than 2000 mg/kg of body weight in both male and female-rats.

Primary dermal irritation

Primary dermal irritation was investigated in male and female New Zealand albino rabbits to evaluate the potential of UC-II to produce irritation after a single topical application. Following application of UC-II, all animals appeared active and healthy. Apart from the dermal irritation noted below, there were no signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior. One hour after patch removal, very slight erythema was observed at all three treated sites. The overall incidence and severity of irritation decreased with time. All animals were free from dermal irritation within 24 h. A summary of Draize primary dermal irritation scoring criteria for dermal reactions and descriptive rating for mean primary dermal irritation index (PDII) is presented in Table 2. Under the conditions of this study, the PDII for UC-II was calculated to be 0.3, thus classifying UC-II to be slightly irritating to the skin (Table 5).

Primary eye irritation

A primary eye irritation test was conducted in New Zealand albino rabbits to determine the potential for UC-II to cause irritation from a single instillation via the ocular route. All animals appeared active and healthy. There were no signs of gross toxicity, adverse pharmacologic effects or abnormal behavior. No corneal opacity or iritis was observed in any treated eye during the study. One hour following UC-II instillation, all treated eyes exhibited conjunctivitis (Table 6). Individual eye irritation scores are presented in Table 6 in accordance with the Draize Scale for scoring Eye Lesions and the Kay and Calandra Scheme for classifying eye irritants (Draize et al. 1944; Kay and Calandra 1962). The overall severity of irritation decreased with time (Table 7). All animals were free of ocular irritation within 48 h. Under the conditions of this study, the maximum mean total score (MMTS) of UC-II powder was determined to be 37.7 (Table 7), classifying UC-II to be moderately irritating to the eye.

Mutagenicity test: Ames' bacterial reverse mutation assay

No toxic effects of UC-II were noted in any of the five tester strains used up to the highest dose group evaluated (with and without metabolic activation). No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with UC-II at any concentration level, in neither the presence nor absence of metabolic activation. Therefore, UC-II did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used, indicating that UC-II is non-mutagenic.

Mutagenicity test: Mouse lymphoma assay

In experiment I with metabolic activation, the relative total growth (RTG) was 108.55% for the highest concentration (2000 μg/ml) evaluated. The highest concentration evaluated without metabolic activation was 2000 μg/ml with an RTG of 83.73%. In experiment II with metabolic activation, the RTG was 90.38% for the highest concentration (2000 μg/ml) evaluated. The highest concentration evaluated without metabolic activation was 440 μg/ml with an RTG of 10.11%.

No biologically relevant increases of mutants were found after treatment with UC-II (with or without metabolic activation) in both experiments I and II. No dose-response relationship was observed. Additionally, in experiments I and II colony sizing showed no clastogenic effects induced by UC-II. Therefore, under the experimental conditions of this study, no evidence of mutagenic activity was detected for UC-II in the L5178Y mouse lymphoma cell line, and UC-II is concluded to be negative for the induction of mutagenicity in this assay.

Dose-dependent 90-day sub-chronic toxicity study

Ophthalmoscopic examinations

Both eyes of all animals were examined by focal illumination and indirect ophthalmoscopy prior to study initiation

Table 5. Summary of primary skin irritation scores (average for three animals).

	Time after patch removal			
	30–60 min	24 h	48 h	72 h
Erythema	1.0	0	0	0
Edema	0	0	0	0
Total (PDI*)	1.0	0	0	0

PDI: Primary dermal irritation = average erythema + average edema.

Table 4. Summary of acute dermal toxicity findings.

	Body weight (g)			Cage-side observations	Necropsy observations
Sex	Initial	Day 7	Day 14	(days 0–14)	(all tissues)
Male	252	299	341	Active and healthy	No gross abnormalities
Male	248	302	352	Active and healthy	No gross abnormalities
Male	239	322	364	Active and healthy	No gross abnormalities
Male	257	314	369	Active and healthy	No gross abnormalities
Male	251	299	349	Active and healthy	No gross abnormalities
Female	196	204	234	Active and healthy	No gross abnormalities
Female	201	218	232	Active and healthy	No gross abnormalities
Female	210	223	240	Active and healthy	No gross abnormalities
Female	211	216	245	Active and healthy	No gross abnormalities
Female	199	225	249	Active and healthy	No gross abnormalities

Table 6. Individual scores for ocular irrigation.

			I. Cornea			II. Iris		III. Conjunctivas				
			A. Opacity	B. Area	(A×B)×5	A. Values	A×5	A. Redness	B. Chemosis	C. Discharge	(A+B+C)×2	Total
Rabbit 3401 Male	Hours	1	1	3	15	1	5	3	2	3[b]	16	36
		24	1[a]	1	5	1	5	3	2	2	14	24
		48	0[a]	4	0	0	0	2	1	1	8	8
		72	0	4	0	0	0	1	0	0	2	2
	Days	4	0	4	0	0	0	0	0	0	0	0
Rabbit 3402 Female	Hours	1	1	3	15	1	5	3	2	3[b]	16	36
		24	1[a]	1	5	1	5	2	2	2	12	22
		48	0[a]	4	0	0	0	2	1	1	8	8
		72	0	4	0	0	0	1	0	0	2	2
	Days	4	0	4	0	0	0	0	0	0	0	0
Rabbit 3402 Female	Hours	1	1	4	20	1	5	3	2	3[b]	16	41
		24	1[a]	2	10	1	5	2	2	2	12	27
		48	0[a]	4	0	0	0	2	1	1	8	8
		72	0	4	0	0	0	1	0	0	2	2
	Days	4	0	4	0	0	0	0	0	0	0	0

[a] 2% ophthalmic fluorescein sodium was used to evaluate the extent or verify the absence of corneal opacity.
[b] Discharge was white in color.

Table 7. Summary of mean scores of severity and reversibility of primary eye irritation study.

Time post-instillation	Severity of irritation
1 h	37.7
24 h	24.3
48 h	8.0
72 h	2.0
4 days	0.0

Maximum Mean Total Score (MMTS) was observed at 1 h post-instillation. See Materials and methods section for details.

and near experimental completion (Day 91). Both eyes of all surviving animals were ophthalmoscopically normal. There was no indication that the test substance, as evaluated, was an ocular toxicant.

Mortality and clinical observations

Two male animals (one in Group 3 and the other Group 4) were found dead on study days 86 and 69, respectively. The animal in Group 3 was active and healthy prior to death and died immediately following the Motor Activity assessment. A cause of mortality could not be definitively determined; however, there was no evidence to suggest that mortality was attributable to test substance administration. Necropsy revealed a distended stomach filled with gas and food and the kidneys appeared enlarged. These observations had no histological correlate and there were no other apparent remarkable findings. The agonal change of congestion was the notable microscopic finding in the adrenal glands, kidneys, liver, and lung.

The animal in Group 4 died as a suspected result of a gavaging error. Prior to death this animal exhibited hypoactivity, hypothermia, moist rales, and irregular respiration accompanied by a red nasal discharge. Macroscopically, the trachea and esophagus were punctured, the thoracic cavity was filled with a white liquid substance, and the lungs were dark red in color. Puncture of the esophagus noted at necropsy was associated microscopically with the presence of hemorrhage, inflammation, and myofiber degeneration at the edges of the puncture wound consistent with an antemortem incident. Puncture of the trachea noted at necropsy was not observed at the time of trimming. Noted microscopic findings associated with the esophageal puncture were marked lung atelectasis, moderate fibrinous inflammation of the lungs involving the pleura, and slight fibrinous inflammation involving the heart (epicardium). Lymphoid depletion noted in lymph nodes, spleen, and thymus was a secondary alteration related to stress/cachexia and was not a primary finding associated with test substance administration.

There were no test substance-related clinical signs in any test group (see Table 3) that were considered to be of toxicological significance. Transient clinical signs included black ocular discharge for one Group 1 (control) male on Days 15–34, one Group 2 (40 mg/kg/day) male on Days 22–35, and one Group 1 female on Day 39. Red ocular discharges for one Group 2 male on Days 43–45; red stained fur for one Group 2 male on Days 50–81 and 84–92, one Group 4 (1000 mg/kg/day) male on Days 50–92, two Group 3 (400 mg/kg/day) females on Days 59–62 and 60–65, respectively, were observed. Red facial stainings for one Group 1 male on Days 71–77, one Group 2 male on Days 42–50 and 82–83, one Group 4 male on Days 39–50, and one Group 3 female on Days 63–70 were noted. Hyperactivity for two Group 3 males on Days 36 and 64 and Day 50, respectively, and one Group 4 male on Day 50 and one Group 2 female on Day 92 was observed. One Group 1 male was noted with a swollen right hindlimb (Days 22–24, 28), hind end impairment (Days 28–42), and swollen foot pads (Days 29–63). One Group 1 male had a wound on the ventral surface of the head on Days 15–27. One Group 1 male had a wound on the right ear on Days 78–92. One Group 3 male had a small scab on the right side of the face on Days 8–18, and one Group 1 female had a small scab on the top of its head on Days 1–19; one Group 4 male exhibited enophthalmos (right eye)

on Days 50–92. One Group 2 male exhibited variable red nasal discharge, reduced fecal volume, ano-genital staining, soft feces, moist rales, hunched posture, and piloerection on Days 53–72. The above findings did not show any adverse effects and did not appear to be test substance-related because they were found across all test groups, including control animals.

Body weight, organ weights, and body weight gain

Weekly body weights for male and female rats at 40, 400, and 1000 mg/kg/day were comparable with control values. Overall (Days 1–92) and mean daily body weight gain for male rats at 40, 400, and 1000 mg/kg/day were comparable with control values (Table 8). Overall (Days 1–92) and mean daily body weight gain for female rats at 40, 400, and 1000 mg/kg/day were generally comparable with control values with the exception that daily body weight gain was decreased during Week 3 for Group 4 females.

There were no changes in individual organ weights (Table 9) or individual organ-to-brain weights (Table 10). The organ-to-body weight ratios were unaffected except that the kidney-to-body weight ratios were significantly decreased in Group 3 males (Table 11). This finding was not associated with any other clinical finding and did not herald any corresponding pathological changes in the high dose animals. Therefore, this change was deemed incidental and of no toxicological interest.

Food consumption and food efficiency

Overall (Days 1–92) and mean daily food consumption for male rats at 40 and 400 mg/kg/day were comparable with control values. Food consumption was decreased for male rats at 1000 mg/kg/day (Group 4) during Weeks 5, 7–11, 13, and overall. Overall and mean daily food consumption for female rats at 40, 400, and 1000 mg/kg/day were generally comparable with control values with the exception of the following statistically significant findings. Food consumption was decreased in females during Weeks 1, 2, and overall at 400 mg/kg/day, and during Weeks 1, 8, and overall at 1000 mg/kg/day.

Overall and mean daily food efficiency for male rats at 40, 400, and 1000 mg/kg/day were comparable with control values. Overall and mean daily food efficiency for female rats at 40, 400, and 1000 mg/kg/day were generally comparable with control values with the exception of the following statistically significant findings. Mean daily food efficiency was decreased during Week 3 for females at 40 mg/kg/day and at 1000 mg/kg/day.

In summary, the oral administration of UC-II led to some dose-related decreases in food consumption in males and females; however, body weight, body weight gain, and food efficiency remained generally unaffected. Reductions in food consumption were considered test substance related and may be of some toxicological interest in light of the pathological findings of nasal turbinate eosinophilia at the high dose (see Clinical Pathology section).

Functional observational battery

In general, the functional behavioral results of the test groups of male and female rats were considered comparable to the control groups. Any decreases in quantitative measurements or increases in incidence of open field measurements were minimal and not associated with a constellation of findings that would support a toxicologically significant behavioral change. In males, these findings included normal (sleeping) postures in 5/10 Group 2 males and 2/10 Group 3 males. Enophthalmos for 1/10 Group 4 males, an inactive/alert activity level for 1/10 Group 2 males, 1/10 Groups 3 males, and 1/10 Group 4 males were observed. A slow reaction to right itself for 1/10 Group 1 males; no approach responses for 1/10 Group 2 males and 2/10 Group 3 males; as well as no tactile responses for 2/10 Group 1 males, 1/10 Group 2 males, 2/10 Group 3 males, and 1/10 Group 4 males were noted. In females, these findings included no tactile responses for 1/10 Group 1 females and 1/10 Group 3 females.

Motor activity

The Motor Activity results of the test groups of male and female rats were considered comparable to the control groups. In general, all groups of animals (including control) exhibited a similar level of movement over all

Table 8. Summary of average weekly body weight.

	Group (male)				Group (female)			
Days	1	2	3	4	1	2	3	4
1	207.2 ± 6.1	207.5 ± 6.9	206.8 ± 6.1	205.7 ± 6.3	160.7 ± 8.1	159.4 ± 7.3	157.7 ± 6.9	161.0 ± 7.0
8	252.2 ± 9.2	251.8 ± 11.7	247.7 ± 9.1	247.3 ± 10.9	182.6 ± 8.6	175.6 ± 11.4	174.3 ± 7.4	175.4 ± 9.1
15	278.5 ± 13.6	277.7 ± 13.8	274.2 ± 10.5	271.1 ± 14.6	195.6 ± 7.4	189.8 ± 12.0	189.9 ± 8.7	191.4 ± 9.4
22	302.6 ± 17.9	305.5 ± 18.5	299.4 ± 11.2	294.3 ± 19.0	215.3 ± 10.5	202.6 ± 14.7	208.8 ± 14.0	202.7 ± 10.8
29	318.3 ± 24.4	322.8 ± 18.7	315.9 ± 12.6	311.8 ± 18.4	223.3 ± 13.4	212.3 ± 15.9	211.7 ± 16.5	211.3 ± 12.5
36	332.6 ± 26.5	336.3 ± 20.9	330.0 ± 12.0	326.3 ± 20.5	225.5 ± 12.6	218.0 ± 13.5	215.2 ± 10.9	215.4 ± 10.9
43	349.7 ± 25.2	351.7 ± 23.8	345.7 ± 13.0	324.8 ± 23.1	234.1 ± 14.4	222.4 ± 16.5	225.2 ± 17.0	224.8 ± 14.5
50	364.2 ± 24.3	367.2 ± 25.2	359.4 ± 15.5	352.4 ± 23.6	241.7 ± 16.2	229.3 ± 15.6	229.6 ± 16.8	233.0 ± 17.5
57	373.2 ± 24.3	368.6 ± 37.2	368.9 ± 17.2	358.5 ± 25.2	246.7 ± 18.7	234.5 ± 18.4	233.7 ± 15.3	233.4 ± 13.6
64	379.6 ± 25.0	377.0 ± 36.8	376.4 ± 18.3	360.1 ± 29.5	249.1 ± 16.0	238.2 ± 17.7	235.9 ± 15.1	237.1 ± 15.8
71	386.3 ± 25.1	386.5 ± 28.1	385.5 ± 17.6	371.0 ± 27.9†	250.5 ± 13.6	241.2 ± 16.4	239.8 ± 14.6	241.7 ± 14.9
78	396.2 ± 28.3	397.6 ± 28.0	394.0 ± 18.5	380.8 ± 32.0†	254.5 ± 15.4	245.2 ± 12.9	244.6 ± 18.5	244.8 ± 14.6
85	400.2 ± 27.9	402.4 ± 28.7	399.2 ± 18.6	388.1 ± 29.8†	256.3 ± 15.2	247.5 ± 15.7	246.7 ± 16.2	246.3 ± 13.7
92	394.9 ± 25.2	398.3 ± 29.9	390.9 ± 19.5†	377.7 ± 26.4†	250.7 ± 12.7	243.0 ± 14.8	240.3 ± 17.1	242.1 ± 15.0

Values are the Mean ± SD ($n = 10$ except for † $n = 9$). No significant difference from control was observed. See Materials and methods section for details.

Table 9. Summary of mean organ weight.

	Group (male)				Group (female)			
Organ	1	2	3	4	1	2	3	4
Adrenals	0.066 ± 0.009†	0.071 ± 0.009	0.067 ± 0.006	0.067 ± 0.011	0.074 ± 0.006	0.072 ± 0.011	0.070 ± 0.004	0.072 ± 0.008
Brain	1.99 ± 0.10	1.98 ± 0.07	1.99 ± 0.07	1.95 ± 0.10	1.85 ± 0.05	1.81 ± 0.06	1.81 ± 0.07	1.85 ± 0.10
Heart	1.31 ± 0.13	1.38 ± 0.11	1.29 ± 0.08	1.29 ± 0.16	0.90 ± 0.09	0.95 ± 0.09	0.89 ± 0.09	0.93 ± 0.09
Kidney	2.90 ± 0.28	2.92 ± 0.19	2.65 ± 0.13	2.79 ± 0.30	1.76 ± 0.11	1.70 ± 0.08	1.72 ± 0.13	1.74 ± 0.18
Liver	10.07 ± 0.87	10.51 ± 0.79	9.85 ± 0.49	9.25 ± 0.83	6.01 ± 0.50	5.99 ± 0.33	5.81 ± 0.34	5.96 ± 0.41
Spleen	0.76 ± 0.10	0.81 ± 0.09	0.75 ± 0.08	0.69 ± 0.08	0.60 ± 0.07	0.62 ± 0.06	0.59 ± 0.08	0.63 ± 0.09
Thymus	0.300 ± 0.054	0.366 ± 0.129	0.313 ± 0.059	0.271 ± 0.062	0.260 ± 0.025	0.243 ± 0.058	0.253 ± 0.038	0.233 ± 0.055
Epididymides	1.490 ± 0.175	1.434 ± 0.222†	1.516 ± 0.119	1.569 ± 0.123	—	—	—	—
Testes	3.87 ± 0.31	3.98 ± 0.23	3.81 ± 0.33	3.80 ± 0.37	—	—	—	—
Ovaries	—	—	—	—	0.139 ± 0.018	0.131 ± 0.017	0.134 ± 0.018	0.147 ± 0.023
Uterus/ Fallopian tubes	—	—	—	—	0.78 ± 0.19	0.65 ± 0.24	0.75 ± 0.50	0.67 ± 0.21

Values are the Mean ± SD ($n = 10$ except for † $n = 9$). No significant difference from control was observed. See Materials and methods section for details.

Table 10. Summary of mean organ-to-brain weight ratios.

	Group (male)				Group (female)			
Organ	1	2	3	4	1	2	3	4
Adrenals	0.030 ± 0.011†	0.036 ± 0.004	0.034 ± 0.004	0.035 ± 0.006	0.040 ± 0.003	0.040 ± 0.006	0.039 ± 0.003	0.039 ± 0.006
Heart	0.66 ± 0.07	0.70 ± 0.06	0.65 ± 0.05	0.66 ± 0.09	0.49 ± 0.05	0.53 ± 0.05	0.49 ± 0.04	0.51 ± 0.05
Kidney	1.45 ± 0.13	1.47 ± 0.09	1.33 ± 0.09	1.43 ± 0.15	0.95 ± 0.07	0.94 ± 0.05	0.95 ± 0.06	0.94 ± 0.07
Liver	5.05 ± 0.40	5.31 ± 0.42	4.95 ± 0.32	4.76 ± 0.50	3.25 ± 0.24	3.31 ± 0.25	3.22 ± 0.18	3.23 ± 0.24
Spleen	0.38 ± 0.06	0.41 ± 0.05	0.38 ± 0.04	0.35 ± 0.05	0.32 ± 0.03	0.35 ± 0.04	0.32 ± 0.04	0.34 ± 0.04
Thymus	0.151 ± 0.026	0.185 ± 0.066	0.157 ± 0.030	0.140 ± 0.034	0.140 ± 0.012	0.134 ± 0.032	0.140 ± 0.021	0.126 ± 0.029
Epididymides	0.747 ± 0.069	0.725 ± 0.120†	0.760 ± 0.040	0.807 ± 0.080	—	—	—	—
Testes	1.94 ± 0.10	2.01 ± 0.14	1.91 ± 0.16	1.95 ± 0.24	—	—	—	—
Ovaries	—	—			0.075 ± 0.009	0.072 ± 0.011	0.074 ± 0.009	0.079 ± 0.011
Uterus/ Fallopian tubes	—	—			0.42 ± 0.10	0.36 ± 0.13	0.42 ± 0.29	0.36 ± 0.10

Values are the Mean ± SD ($n = 10$ except for † $n = 9$). No significant difference from control was observed. See Materials and methods section for details.

Table 11. Summary of mean organ-to-body weight ratios.

	Group (male)				Group (female)			
Organ	1	2	3	4	1	2	3	4
Adrenals	0.178 ± 0.029†	0.188 ± 0.018	0.180 ± 0.014	0.190 ± 0.038	0.315 ± 0.031	0.317 ± 0.064	0.310 ± 0.031	0.316 ± 0.046
Brain	5.34 ± 0.31	5.27 ± 0.45	5.38 ± 0.31	5.49 ± 0.51	7.86 ± 0.49	7.94 ± 0.56	7.99 ± 0.41	8.11 ± 0.38
Heart	3.48 ± 0.22	3.67 ± 0.41	3.49 ± 0.28	3.62 ± 0.28	3.81 ± 0.29	4.16 ± 0.34	3.91 ± 0.30	4.09 ± 0.41
Kidney	7.72 ± 0.46	7.76 ± 0.62	7.14 ± 0.34*	7.81 ± 0.46	7.47 ± 0.61	7.43 ± 0.50	7.58 ± 0.35	7.62 ± 0.56
Liver	26.84 ± 0.94	27.91 ± 1.91	26.55 ± 0.66	25.92 ± 0.77	25.45 ± 1.43	26.21 ± 1.54	25.65 ± 1.21	26.11 ± 1.50
Spleen	2.04 ± 0.26	2.17 ± 0.30	2.02 ± 0.17	1.92 ± 0.14	2.55 ± 0.32	2.73 ± 0.29	2.58 ± 0.28	2.77 ± 0.38
Thymus	0.801 ± 0.134	0.974 ± 0.374	0.844 ± 0.163	0.766 ± 0.194	1.099 ± 0.086	1.064 ± 0.264	1.119 ± 0.167	1.019 ± 0.229
Epididymides	3.992 ± 0.510	3.876 ± 0.777†	4.089 ± 0.305	4.421 ± 0.444	—	—	—	—
Testes	10.35 ± 0.75	10.59 ± 0.83	10.29 ± 1.11	10.66 ± 0.90	—	—	—	—
Ovaries	—	—	—	—	0.589 ± 0.090	0.570 ± 0.061	0.591 ± 0.059	0.641 ± 0.089
Uterus/ Fallopian tubes	—	—	—	—	3.30 ± 0.90	2.89 ± 1.21	3.32 ± 2.29	2.95 ± 0.86

Values are the Mean ± SD ($n = 10$ except for † $n = 9$). * Statistically significant different from control value ($p < 0.05$). See Materials and methods section for details.

intervals. No statistical differences were noted in any male or female group compared to their corresponding control (Table 12).

Clinical pathology

Hematology. Absolute platelet count (PLT) was significantly decreased in males administered 40 mg/kg/day compared with control animals (86% of control). This change in mean hematology parameters was not adverse and not considered related to exposure to the test substance because the pathological changes did not occur in a dose-related pattern and because they were not accompanied by any other corresponding clinical- or histopathological change.

Table 12. Summary of motor activity assessment.

	Group (male)				Group (female)			
Interval	1	2	3	4	1	2	3	4
1	158.6 ± 30.74	152.5 ± 25.96	154.2 ± 19.93	179.9 ± 41.01	163.8 ± 26.94	168.8 ± 29.90	153.6 ± 23.29	156.2 ± 14.52
2	93.9 ± 19.3	84.5 ± 20.7	93.3 ± 28.1	102.8 ± 21.9	95.2 ± 26.1	98.4 ± 28.4	79.8 ± 12.9	100.4 ± 15.82
3	63.3 ± 14.3	65.7 ± 22.6	72.9 ± 25.5	80.7 ± 20.3	66.1 ± 17.0	87.0 ± 28.0	61.3 ± 22.5	78.1 ± 17.4
4	64.8 ± 23.4	67.9 ± 25.3	70.5 ± 22.6	62.1 ± 15.3	67.8 ± 31.6	62.4 ± 25.6	59.9 ± 18.2	61.2 ± 19.9
5	62.8 ± 14.4	50.2 ± 12.2	60.9 ± 18.9	54.3 ± 27.1	51.6 ± 14.8	62.5 ± 24.5	56.3 ± 12.5	52.4 ± 24.8
6	63.3 ± 15.2	59.7 ± 27.0	59.0 ± 23.1	48.6 ± 26.6	72.8 ± 24.8	57.1 ± 28.9	44.5 ± 10.1	57.0 ± 13.4

Values are the Mean ± SD. No significant difference from control was observed. See Materials and methods section for details.

Absolute eosinophil concentration (AEOS) was significantly increased in males administered 1000 mg/kg/day (200% of control). Absolute eosinophil concentration was also significantly increased in high dose females in a generalized dose-related response. However, values did not reach the level of statistical significance due to high variability within the group. Two females, in particular, showed high eosinophil levels, and this contributed to the overall increase in the group. Given that this finding occurred in more than one animal within the group and occurred as a generalized increase in all the males in Group 4, a test-substance related effect could not be discounted in females.

In addition to the above findings, one high dose female displayed detectable concentrations of absolute neutrophil band (ABAN). This finding, while appearing non-adverse, might be associated with an individual generalized granulocytic increase in response to test substance administration at the high dose.

Coagulation. There were no treatment-related or statistically significant effects in coagulation parameters.

Clinical biochemistry. There were no adverse changes in clinical biochemistry parameters in male or female rats (Table 13). The following statistically significant changes in mean clinical biochemistry results were not adverse and not considered related to exposure to the test substance because they were not dose-related and because they were not accompanied by any other corresponding clinical- or histopathological change. An increase in the aspartate aminotransferase (AST) concentration in males administered 40 mg/kg/day and females administered 400 mg/kg/day (113 and 115% of control, respectively) was observed. A decrease in sorbitol dehydrogenase (SDH) in females administered 40 and 400 mg/kg/day (66 and 75% of control, respectively) occurred. Both aspartate aminotransferase and sorbitol dehydrogenase are hepatocytic enzymes, their leakage suggestive of liver injury. However, only SDH is liver-specific, and is often accompanied by significant loss of liver mass. Given the absence of dose-dependent changes, uncorrelative with any microscopic alterations as well as the small magnitude of the change, there was no evidence to suggest that these changes were toxicologically relevant to test substance administration.

Urinalysis. There were no treatment-related or statistically significant effects in urinalysis parameters.

Serology. There were no detectable titers against the pathogens and antigens tested. In conclusion, there were no adverse changes in coagulation, clinical chemistry, or urinalysis parameters in male or female rats administered UC-II. The statistically significant increase in eosinophil concentration in high dose males, with increases in high dose females were considered related to exposure to the test substance because this dose-related change was accompanied by potentially adverse histopathological change in the nasal cavity of both male and female high dose animals.

Sacrifice, macroscopic observations, and histopathology. There were no UC-II related macroscopic findings at scheduled sacrifice, and mortality occurring prematurely was deemed unrelated to test substance administration. At termination, test substance-related microscopic findings were observed involving the nasal turbinates in males and females at 1000 mg/kg/day UC-II. An increase in the incidence and intensity of several findings involving the respiratory epithelium were noted in males and females at 1000 mg/kg/day UC-II as compared to their respective controls. Findings included goblet cell hypertrophy/hyperplasia, eosinophilic infiltrates, acute inflammation, and the presence of eosinophilic cytoplasmic droplets. The incidence and intensity of these microscopic findings are presented in Table 14. The presence of eosinophilic droplets in the nasal turbinates of mice has been described as a non-adverse, adaptive response. Similarly, in this instance, their presence was deemed secondary to the other morphologic alterations described above for the nasal turbinates. There were statistically significant increases in absolute eosinophil counts for males at 1000 mg/kg/day and a non-statistically significant increase in mean absolute eosinophil counts for females at 1000 mg/kg/day. These hematologic alterations are likely associated with the eosinophil infiltrates in the nasal turbinates, which may reflect a test substance-related hypersensitivity reaction at the highest dosage tested.

Microscopic findings unrelated to the test-substance administration include: sporadic alterations involving the esophagus attributable to repeated gavage procedures, such as minimal-to-moderate esophageal changes included myofiber degeneration as well as fibroplasia, hemorrhage, inflammation, and pigmented macrophages (consistent with hemosiderin and resolving hemorrhage) involving the esophageal wall. In addition, sporadic findings of minimal chronic inflammation and necrosis involving the Harderian glands were attributable to sequelae of end of study orbital sinus bleeds. The remaining findings were incidental and most commonly developmental,

Table 13. Mean clinical biochemistry values.

	Group (male)				Group (female)			
Parameter (Units)	1	2	3	4	1	2	3	4
Aspartate Aminotransferase (AST, U/L)	91 ± 27	103 ± 15*	96 ± 15	97 ± 19	85 ± 7	97 ± 11	98 ± 17*	85 ± 9
Alanine Aminotransferase (ALT, U/L)	44 ± 7	49 ± 5	44 ± 5	50 ± 10	36 ± 4	41 ± 6	41 ± 6	37 ± 3
Sorbitol Dehydrogenase (SDH, U/L)	9.2 ± 2.5	8.6 ± 3.7	8.2 ± 2.7	9.2 ± 1.7	10.8 ± 2.4	7.1 ± 2.1*	8.1 ± 2.8*	8.6 ± 1.7
Alkaline Phosphatase (ALKP, U/L)	126 ± 32	137 ± 22	139 ± 34	135 ± 27	103 ± 30	103 ± 27	104 ± 23	88 ± 18
Total Bilirubin (BILI, mg/dL)	0.14 ± 0.02	0.14 ± 0.03	0.14 ± 0.003	0.16 ± 0.03	0.18 ± 0.02	0.20 ± 0.04	0.19 ± 0.03	0.18 ± 0.03
Blood Urea Nitrogen (BUN, mg/dL)	21 ± 3	21 ± 3	20 ± 1	21 ± 5	20 ± 2	21 ± 3	22 ± 4	23 ± 2
Creatinine (CREA, mg/dL)	0.29 ± 0.03	0.31 ± 0.03	0.31 ± 0.02	0.31 ± 0.04	0.39 ± 0.04	0.39 ± 0.06	0.41 ± 0.05	0.39 ± 0.04
Cholesterol (CHOL, mg/dL)	79 ± 10	82 ± 10	80 ± 9	80 ± 8	90 ± 18	84 ± 10	84 ± 13	85 ± 18
Triglycerides (TRIG, mg/dL)	49 ± 9	45 ± 8	45 ± 12	38 ± 8	28 ± 5	31 ± 6	28 ± 6	27 ± 7
Glucose (GLUC, mg/dL)	159 ± 24	160 ± 36	155 ± 23	160 ± 30	119 ± 15	120 ± 12	125 ± 14	116 ± 15
Total protein (TP, g/dL)	6.3 ± 0.2	6.3 ± 0.3	6.4 ± 0.2	6.4 ± 0.3	6.5 ± 0.3	6.8 ± 0.5	6.8 ± 0.2	6.8 ± 0.2
Albumin (ALB, g/dL)	3.2 ± 0.2	3.2 ± 0.1	3.2 ± 0.1	3.3 ± 0.2	3.5 ± 0.2	3.6 ± 0.1	3.5 ± 0.2	3.6 ± 0.2
Globulin (GLOB, g/dL)	3.1 ± 0.2	3.2 ± 0.3	3.1 ± 0.2	3.1 ± 0.2	3.0 ± 0.2	3.2 ± 0.4	3.3 ± 0.3	3.2 ± 0.2
Calcium (CALC, mg/dL)	9.5 ± 0.5	9.6 ± 0.5	9.7 ± 0.2	9.6 ± 0.3	9.8 ± 0.4	9.9 ± 0.5	9.9 ± 0.3	10.0 ± 0.3
Inorganic Phosphorus (IPHS, mg/dL)	6.3 ± 0.7	6.6 ± 1.0	6.5 ± 0.5	6.5 ± 0.5	6.0 ± 0.9	5.8 ± 0.7	6.2 ± 0.5	5.5 ± 0.5
Sodium (NA, mmol/L)	144.6 ± 6.0	144.8 ± 3.9	145.0 ± 6.6	145.0 ± 4.1	146.1 ± 5.5	145.4 ± 7.7	146.7 ± 4.4	148.0 ± 7.6
Potassium (K, mmol/L)	6.06 ± 0.60	5.99 ± 0.75	5.86 ± 0.47	6.14 ± 0.32	5.17 ± 0.44	5.37 ± 0.45	5.33 ± 0.47	5.32 ± 0.54
Chloride (CL, mmol/L)	103.5 ± 3.1	103.8 ± 3.2	104.2 ± 4.6	103.2 ± 1.7	105.8 ± 2.6	105.3 ± 4.4	105.7 ± 3.1	107.1 ± 4.5

Values are the mean ± SD ($n \geq 8$). * Statistically significant different from control values ($p < 0.05$).

Table 14. Incidence and severity of microscopic nasal turbinate findings.

Group	1		3		4	
Dose volume (mg/kg/day)	0		400		1000	
Sex	Male	Female	Male	Female	Male	Female
Goblet cell hypertrophy/hyperplasia: respiratory epithelium	1	0	0	1	9	9
Grade 1	0	0	0	0	0	0
Grade 2	1	0	0	1	5	8
Grade 3	0	0	0	0	4	1
Eosinophil infiltrates: respiratory epithelium	1	0	1	1	9	9
Grade 1	1	0	1	1	5	2
Grade 2	0	0	0	0	4	7
Acute inflammation: respiratory epithelium	0	0	0	0	4	1
Grade 1	0	0	0	0	3	1
Grade 2	0	0	0	0	1	0
Eosinophil droplets: respiratory epithelium cytoplasmic	0	0	1	1	9	7
Grade 1	0	0	0	1	4	4
Grade 2	0	0	1	0	5	3

See Materials and methods section for details.

inflammatory, or degenerative changes that can be seen in the age and strain of rat used in this study. Examples included, but were not limited to, nephropathy, pulmonary alveolar histiocytosis, pituitary gland cyst, and ectopic thymus in thyroid gland. UC-II related microscopic findings were observed involving the respiratory epithelium of the nasal turbinates in males and females at 1000 mg/kg/day UC-II. Salient microscopic observations included eosinophil infiltrates, goblet cell hypertrophy and hyperplasia, and acute inflammation. Therefore, under the conditions of this study, the anatomic pathology no-observed-adverse-effect level (NOAEL) for UC-II was 400 mg/kg/day following daily oral gavage to male and female Sprague-Dawley rats for at least 90 days.

Discussion

Given that OA is the most prevalent form of arthritis and that the number of persons affected with OA will increase significantly in the near future, finding alternative, safer pharmacological therapies for OA is of considerable importance. With the continued growth of the elderly population in the US, OA is becoming a major medical and financial concern. In the last few years, various nutritional supplements including chondroitin, glucosamine, avocado/soybean unsaponifiables, and diacerein have emerged as new treatment options for osteoarthritis. Among these nutraceuticals, the efficacy of UC-II was repeatedly demonstrated in animal (Deparle et al. 2005; D'Altilio et al. 2007; Peal et al. 2007; Bagchi et al. 2008a; 2009; Gupta et al. 2009a; b) and human (Bagchi et al. 2008b; Crowley et al. 2009) studies without any significant adverse events.

The current study demonstrated the broad-spectrum safety of UC-II in animals over the dose levels and routes of administration tested. Acute oral toxicity did not reveal any significant changes for all examined tissues. Based on these results, the oral LD_{50} of UC-II was concluded to be > 5000 mg/kg in female rats. Acute dermal toxicity study conducted with a single 2000 mg/kg dose of UC-II applied directly to the skin of male and female rats for 24 h revealed no dermal irritation, adverse pharmacological effects, or abnormal behavior. Based on these results, the acute dermal LD_{50} of UC-II was > 2000 mg/kg. The primary dermal irritation assay using a single 1000 mg dose of UC-II applied directly to the skin of rabbits for 4 h caused an initial redness of the skin. The overall incidence and severity of irritation decreased with time and irritation completely subsided by 24 h. Based on these results, UC-II was classified as slightly irritating to the skin. There were no other signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior. Primary eye irritation was tested in rabbits using a single dose of 60 mg. One hour after UC-II application, treated eyes exhibited corneal opacity, iritis, and positive conjunctivitis. The overall incidence and severity of irritation decreased gradually with time. All animals were free of ocular irritation within 96 h. Based on these results, UC-II was classified as moderately irritating to the eye.

Ames' Bacterial Reverse Mutation Assay using five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537, and TA102) was used to evaluate the mutagenic potential of UC-II in the presence and absence of metabolic activation. UC-II was determined to be non-mutagenic. Cell gene mutation assay in mouse lymphoma cells was conducted to test the mutagenic potential of UC-II in the L5178Y mouse lymphoma cell line. UC-II did not induce mutagenic effects either with or without metabolic activation.

The results from the 90-day sub-chronic toxicity study did not show any adverse effects in individual body weight or individual organ weight after 90 days of UC-II administration in increasing doses. No significant changes in organ-to-body weight ratios were observed except for the kidney-to-body weight ratio, which was significantly decreased in Group 3 males. This finding was not associated with any other clinical findings, and did not indicate any corresponding pathologic changes in the high dose animals. Therefore, this change was deemed incidental and of no toxicological interest. Mortality of a single Group 3 male and a single Group 4 male were not associated with test substance administration. Test substance-related microscopic findings were observed involving the respiratory epithelium of the nasal turbinates in males and females at 1000 mg/kg/day UC-II. Salient microscopic observations included eosinophil infiltrates, goblet cell hypertrophy and hyperplasia, and acute inflammation. Therefore, under the conditions of this study, the anatomic pathology no-observed-adverse-effect level (NOAEL) for UC-II was 400 mg/kg/day following daily oral gavage to male and female Sprague-Dawley rats for at least 90 days.

Overall, results from the current study combined with the animal (Deparle et al. 2005; D'Altilio et al. 2007; Peal et al. 2007; Bagchi et al. 2008a; 2009; Gupta et al. 2009a; b) and human (Bagchi et al. 2008b; Crowley et al. 2009) data demonstrate the broad-spectrum safety of UC-II.

Declaration of interest

This study was supported by a research grant from InterHealth Nutraceuticals Inc. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, Christy W, Cooke TD, Greenwald R, Hochberg M, Howell D, Kaplan D, Koopman W, Longley III S, Mankin H, McShane DJ, Medsger Jr T, Meenan R, Mikkelsen W, Moskowitz R, Murphy W, Rothschild B, Segal M, Sokoloff L, Wolfe F. 1986. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis Rheum 29:1039–1049.

Ames BN, McCann J, Yamasaki E. 1977: Methods for the Salmonella Mutagenicity Test. In: Kilbey BJ, editor. Handbook of mutagenicity test procedures. Amsterdam: Elsevier. pp 1–17.

Bagchi M, Gupta RC, Lindley J, Barnes M, Canerdy TD, Goad JT, Bagchi D. 2009. Suppression of Arthritic Pain in Dogs by Undenatured Type-II Collagen (UC-II) Treatment Quantitatively Assessed by Ground Force Plate Dresden, Germany: EUROTOX.

Bagchi M, Lau FC, Bagchi D. 2008b. Beneficial effects of oral administration of undenatured type II collagen on osteoarthritis: a human clinical trial. Am Coll Nutr, 27: 603.

Bagchi M, Skaggs P, Gupta RC, Canerdy TD, Goad JT, Barnett D, Wegford K, Burke R, Bagchi D. 2008a Therapeutic Efficacy of Undenatured Type II Collagen (UC-II) In Comparison To Glucosamine Plus Chondroitin in Arthritic Horses San Diego, CA: FASEB. pp LB28.

Barnett ML, Combitchi D, Trentham DE. 1996. A pilot trial of oral type II collagen in the treatment of juvenile rheumatoid arthritis. Arthritis Rheum 39:623–628.

Barnett ML, Kremer JM, St Clair EW, Clegg DO, Furst D, Weisman M, Fletcher MJ, Chasan-Taber S, Finger E, Morales A, Le CH, Trentham DE 1998. Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo-controlled trial. Arthritis Rheum 41:290–297.

Berenbaum F. 2008. New horizons and perspectives in the treatment of osteoarthritis. Arthritis Res Ther 10(Suppl 2):S1

Bitton R. 2009. The economic burden of osteoarthritis. Am J Manag Care 15:S230–S235.

Clive D. 1983. Viable chromosomal mutations affecting the TK locus in L5178Y/TK+/− mouse lymphoma cells: the other half of the assay. Ann NY Acad Sci 407:253–257.

Clive D, McCuen R, Spector JF, Piper C, Mavournin KH. 1983. Specific gene mutations in L5178Y cells in culture. Mutat Res 115:225–251.

Clive D, Spector JF. 1975. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutat Res 31:17–29.

Crowley DC, Lau FC, Sharma P, Evans M, Guthrie, N., Bagchi, M. Bagchi, D., Dey, D. K. Raychaudhuri, S. P. 2009. Safety and efficacy of undenatured type II collagen in the treatment of osteoarthritis of the knee: a clinical trial. Int J Med Sci 6:312–321.

D'Altilio M, Peal A, Alvey M, Simms C, Curtsinger A, Gupta RC, Canerdy TD, Goad JT, Bagchi M, Bagchi D. 2007. Therapeutic efficacy and safety of undenatured type II collagen singly or in combination with glucosamine and chondroitin in arthritic dogs. Toxicol Mech Methods 17:189–196.

Deparle LA, Gupta RC, Canerdy TD, Goad JT, D'Altilio M, Bagchi M, Bagchi D. 2005. Efficacy and safety of glycosylated undenatured type-II collagen (UC-II) in therapy of arthritic dogs. J Vet Pharmacol Ther 28:385–390.

Draize JH, Woodward G, Calvery HO. 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membrane. J Pharm Exp Ther 82:377–390.

FDA. 1987.Good Laboratory Practices (GLP) for Non-Clinical Laboratory Studies. In: Services. D. o. H. a. H. Washington, DC. US Food and Drug Administration. pp297-310.

Felson DT. 2009. Developments in the clinical understanding of osteoarthritis. Arthritis Res Ther 11:203.

Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, Kington RS, Lane NE, Nevitt MC, Zhang Y, Sowers M, McAlindon T, Spector TD, Poole AR, Yanovski SZ, Ateshian G, Sharma L, Buckwalter JA, Brandt KD, Fries JF. 2000. Osteoarthritis: new insights. Part 1: the disease and its risk factors. Ann Intern Med 133:635–646.

Goggs R, Vaughan-Thomas A, Clegg PD, Carter SD, Innes JF, Mobasheri A, Shakibaei M, Schwab W, Bondy CA. 2005. Nutraceutical therapies for degenerative joint diseases: a critical review. Crit Rev Food Sci Nutr 45:145–164.

Gupta RC, Canerdy TD, Skaggs P, Stocker A, Zyrkowski G, Burke R, Wegford K, Goad JT, Rohde K, Barnett D, DeWees, W, Bagchi M, Bagchi D. 2009a. Therapeutic efficacy of undenatured type-II collagen (UC-II) in comparison to glucosamine and chondroitin in arthritic horses. J Vet Pharmacol Ther 32:577–584.

Gupta RC, Lindley J, Barnes M, Minniear J, Goad JT, Canerdy TD, Bagchi M, Bagchi D. 2009b. Pain reduction measured by ground force plate in arthritic dogs treated with type-II collagen Baltimore, MD: Society of Toxicology.

Helmick CG, Felson DT, Lawrence RC, Gabriel S, Hirsch R, Kwoh CK, Liang MH, Kremers HM, Mayes MD, Merkel PA, Pillemer SR, Reveille JD, Stone JH. 2008. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part I. Arthritis Rheum 58:15–25.

Kay JH, Calandra JC. 1962. Interpretation of eye irritation tests. J Soc Cos Chem 13:281–289.

Lawrence RC, Felson DT, Helmick CG, Arnold LM, Choi H, Deyo RA, Gabriel S, Hirsch R, Hochberg MC, Hunder GG, Jordan JM, Katz JN, Kremers HM, Wolfe F. 2008. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. Arthritis Rheum 58:26–35.

Maron DM, Ames BN. 1983. Revised methods for the Salmonella mutagenicity test. Mutat Res 113:173–215.

Mitchell AD, Auletta AE, Clive D, Kirby PE, Moore MM, Myhr BC. 1997. The L5178Y/tk+/− mouse lymphoma specific gene and chromosomal mutation assay a phase III report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat Res 394:177–303.

OECD. 1998. Paris: Committee, C. G. a. M.Principles of Good Laboratory Practice Organization for Economic Cooperation and Development.

Peal A, D'Altilio M, Simms C, Alvey M, Gupta RC, Goad JT, Canerdy TD, Bagchi M, Bagchi D. 2007. Therapeutic efficacy and safety of undenatured type-II collagen (UC-II) alone or in combination with (-)-hydroxycitric acid and chromemate in arthritic dogs. J Vet Pharmacol Ther 30:275–278.

Sarzi-Puttini P, Cimmino MA, Scarpa R, Caporali R, Parazzini F, Zaninelli A, Atzeni F, Canesi B. 2005. Osteoarthritis: an overview of the disease and its treatment strategies. Semin Arthritis Rheum 35:1–10.

Trentham DE. 1984. Immunity to type II collagen in rheumatoid arthritis: a current appraisal. Proc Soc Exp Biol Med 176:95–104.

Trentham DE. 1996. Evidence that type II collagen feeding can induce a durable therapeutic response in some patients with rheumatoid arthritis. Ann NY Acad Sci 778:306–314.

Trentham DE, Dynesius-Trentham RA, Orav EJ, Combitchi D, Lorenzo C, Sewell KL, Hafler DA, Weiner HL 1993. Effects of oral administration of type II collagen on rheumatoid arthritis. Science 261:1727–1730.

Trentham DE, Halpner AD, Trentham RA, Bagchi M, Kothari SC, Preuss HG, Bagchi D. 2001. Use of undenatured type II collagen in the treatment of rheumatoid arthritis. Clin Pract Altern Med 2: 254–259.

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Market: USA and Internet

Myogenix

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Market: USA and Internet

Douglas Labs

Product: RheumaShield™

Contains: UC-II®, Devil's Claw Extract and Bromelain

Market: USA and Internet

Sun Naturals
(Arnet Pharmaceuticals)

Product: UC-II®

Contains: UC-II® only

Market: USA and Internet

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Product: Joint-UC-II

Contains: UC-II®

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