INDUCTION OF IMMUNE TOLERANCE TO HUMAN TYPE I COLLAGEN IN PATIENTS WITH SYSTEMIC SCLEROSIS BY ORAL ADMINISTRATION OF BOVINE TYPE I COLLAGEN

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Objective. To determine whether oral tolerance to type I collagen (CI) could be induced in patients with systemic sclerosis (SSc).

Methods. Twenty adult patients with limited or diffuse SSc were enrolled in a study to receive 0.1 mg of solubilized native bovine CI daily for 1 month, followed by 0.5 mg daily for 11 months. Peripheral blood mononuclear cells (PBMC) were obtained from the patients and cultured with human α1(I) and α2(I) chains, before and after CI treatment. Culture supernatants were analyzed for levels of interferon-γ (IFNγ) and interleukin-10 (IL-10). Sera obtained before and after treatment were analyzed for levels of soluble IL-2 receptor (sIL-2R). Although this study was not intended to assess the clinical efficacy of oral CI administration in SSc, selected measures of disease severity and organ involvement were evaluated.

Results. Oral administration of CI to SSc patients induced significant reductions in levels of IFNγ and IL-10 in α1(I)- and α2(I)-stimulated PBMC culture supernatants, indicating that T cell immunity to CI was decreased by this treatment. Serum levels of sIL-2R also decreased significantly after oral CI treatment, suggesting a reduction in T cell activation. Significant improvements occurred in the modified Rodnan skin thickness score and the modified Health Assessment Questionnaire after 12 months of oral CI in this open trial. The lung carbon monoxide diffusing capacity improved statistically and showed a trend toward clinically significant improvement.

Conclusion. Oral administration of bovine CI to patients with diffuse or limited SSc induces a reduction in T cell reactivity to human CI, appears to be well tolerated, and does not worsen the disease. Further evaluation of oral tolerance to CI in patients with SSc is justified to determine whether it has therapeutic efficacy.

Type I collagen (CI) is the most abundant of all collagens in humans (1). It is present in blood vessels, skin, lungs, heart, kidneys, and intestines, all of which are affected in systemic sclerosis (SSc) (1). CI is a heterotrimer molecule composed of two identical α1(I) chains and one α2(I) chain (1). Each α chain contains 1,014 amino acid residues (1). Human and bovine CI have ~92% homology at the amino acid level (2,3). Evidence for cellular immunity to CI in SSc patients was first demonstrated by our group in 1976 (4) and has been confirmed by other investigators (5,6). We found that peripheral blood mononuclear cells (PBMC) from 92% of SSc patients produce chemotactic cytokines when cultured with CI, whereas only 8% of PBMC from healthy subjects do so (4). Hawrylko et al (5) also showed that peripheral blood CD4+ T cells from patients with SSc produce interleukin-2 (IL-2) in a dose-dependent manner in response to stimulation with human CI, while those from healthy subjects do not.

A major portion (approximately one-third) of the body’s immune cells reside in the gut-associated lymphoid tissue (GALT) (7). The GALT is particularly effective in mounting a tolerogenic response to ingested soluble proteins (7,8). This process, called oral toler-
ance, has been repeatedly demonstrated in laboratory animals. For example, when mouse strains susceptible to experimental allergic encephalomyelitis (EAE) after systemic immunization with myelin basic protein (MBP) are fed MBP prior to immunization, they develop less EAE or no EAE compared with placebo-fed MBP-immunized controls (9).

The mechanisms that mediate oral tolerance include active cellular suppression (regulatory T cells), clonal anergy, and clonal deletion (10–12). The particular dose of antigen and the frequency of feeding determine which mechanism(s) predominates (10,12). Multiple oral feedings of low-dose soluble antigen favor development of regulatory CD4+ T cells that secrete Th2 cytokines, such as IL-4 and IL-10, and transforming growth factor β1 (TGFβ1)–secreting T cells (Th3 cells) (10,12). These regulatory T cells migrate to peripheral sites throughout the body, and when they encounter the antigen to which they are tolerized, they collectively secrete IL-4, IL-10, and TGFβ1, which can down-regulate Th1 CD4 cells reacting to a variety of antigens, a process called “bystander suppression” (10,13,14).

Since many of the antigens that are involved in human autoimmune diseases are unknown, it is theoretically possible that, by feeding low doses of antigen from the organs or tissues that are the target of autoimmune attack, T cell responses to other autoantigens perpetuating the disease can be down-regulated. CI qualifies as a candidate oral tolerance antigen in SSc, in that it is present in all of the target organs. Since most SSc patients exhibit sensitization to CI (1,4–6), as manifested by cytokine production by PBMC during culture with CI or constituent α1 and α2 chains, successful tolerization to CI after it has been orally administered to SSc patients can be assessed by determining whether there are decreases in cytokine production by PBMC cultured with CI α chains. The present phase I study was undertaken to determine whether daily administration of oral bovine CI to patients with SSc would result in down-regulation of the immune response to human CI.

**PATIENTS AND METHODS**

**Patient recruitment and characteristics.** This study was approved by the Institutional Review Board at The University of Tennessee Health Science Center. Patients were recruited from University of Tennessee and community rheumatology practices in Memphis. Inclusion criteria were as follows: age ≥18 years; diagnosis of limited or diffuse SSc by the criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) (15); and patient’s PBMC demonstrated reactivity to bovine CI, as defined by the production of IL-10 (≥2 times baseline production) when cultured with native bovine CI. Patients taking D-penicillamine, captopril, or calcium channel blockers were required to be receiving stable doses of these agents for at least 3 months prior to enrollment. The maximum allowable dosage for D-penicillamine was 750 mg/day. Patients taking corticosteroids were required to be receiving a stable dose for at least 1 month prior to enrollment; the maximum allowable dosage was 10 mg/day of prednisone equivalent.

Patients were excluded from the study for the following reasons: inability to render an informed consent in accordance with institutional guidelines; receiving another investigational drug (excluding D-penicillamine) within 90 days of study initiation; a concurrent serious medical condition that, in the opinion of the investigators, made the patient inappropriate for the study; an SSc-like illness associated with environmental, ingested, or injected agents, such as L-tryptophan, tainted rapeseed oil, vinyl chloride, or bleomycin; morphea, linear scleroderma, or eosinophilic fasciitis; a positive pregnancy test; use in the previous 3 months of cyclophosphamide, cyclosporin A, methotrexate, or azathioprine; allergy to beef; or malabsorption syndrome.

**Design and duration of the study.** The study was an open-label trial to determine whether oral CI treatment would down-regulate PBMC cytokine production when cultured with α1(I) and α2(I). Patients received 0.1 mg/day of solubilized bovine CI for 1 month, followed by 0.5 mg/day for 11 months. Collagen was solubilized in 0.1 M acetic acid and aliquoted into individual-dose vials. Patients kept the vials refrigerated. Each morning, the patient added 1 vial of the CI preparation to 4–6 ounces of cold orange juice and drank it just before eating breakfast. Patient compliance was monitored by counting the numbers of empty and full vials returned at each visit.

**Concomitant medication.** Patients were not allowed to increase dosages of D-penicillamine, captopril, calcium channel blockers, or corticosteroids during the study. Patients were dropped from the study if increases in any of these medications were deemed medically necessary by their primary physicians.

**Clinical measurements.** Significant clinical responses were not expected due to the small study size and the variability in disease classification, manifestations, and duration. However, the following measures of disease severity and organ involvement were evaluated: modified Rodnan skin thickness scores (MRSS) (16) at 0, 1, 2, 3, 6, 9, and 12 months; pulmonary function tests (PFTs; spirometry) with measurement of the diffusing capacity for carbon monoxide (DLCO); performed by the same personnel using the same equipment) at 0, 3, 6, 9, and 12 months; serum creatinine levels at 0, 1, 3, 6, 9, and 12 months; and the modified Health Assessment Questionnaire (M-HAQ) (17,18) at 0, 3, 6, 9 and 12 months.

**Microculture of SSc PBMC with α1(I) and α2(I).** Briefly, before and after 3, 6, and 12 months of oral bovine CI treatment, PBMC were isolated by Ficoll-Hypaque density gradient centrifugation and set up in culture in 48-well tissue culture plates (2 × 10^5 cells in 0.5 ml of RPMI 1640 containing 100 units/ml of penicillin, 100 µg/ml of streptomycin, 2 mM l-glutamine, 20 mM HEPES, and 5% fetal calf serum). Cultures were set up with 50 µg/ml each of purified bovine α1(I) and α2(I) chains, and phytohemagglutinin (PHA; 10 µg/ml) and phosphate buffered saline (PBS) as controls in duplicate wells. After 5 days of culture, supernatants from duplicate
wells were pooled, harvested by centrifugation, and frozen at −70°C until assayed for cytokine levels (within 30 days).

Measurement of cytokines in serum and PBMC supernatants. After screening for several cytokines in supernatants from SSc PBMC cultured with α1(I) and α2(I), we found that most SSc patients produced increased amounts of interferon-γ (IFNγ) and IL-10 protein, as measured by enzyme-linked immunosorbent assay (ELISA). These cytokines were subsequently measured in all culture supernatants.

IFNγ and IL-10 levels were measured by commercial ELISA (R&D Systems, Minneapolis, MN) in supernatants harvested from microcultures of SSc PBMC stimulated by PHA, α1(I), and α2(I), and PBMC plus PBS as a control for background cytokine production. A positive response to α1(I) or α2(I) was arbitrarily defined as IL-10 or IFNγ levels in α1(I)- or α2(I)-stimulated PBMC culture supernatants that were ≥2 times the respective cytokine level in the PBMC plus PBS control supernatant. Soluble IL-2 receptor (sIL-2R) levels were measured by ELISA (R&D Systems) in sera obtained before and after 12 months of oral CI treatment. All samples were tested in duplicate.

Measurement of T cell subsets by flow cytometry. Isolated PBMC obtained from samples taken at 0 and 6 months of oral CI treatment were reacted with a panel of monoclonal antibodies that recognize T cell–specific markers CD4+, CD8+, CD3+DR+, CD4+CD45+RA+, CD4+CD45+RO+, CD8+CD45+RA+, CD8+CD45+RO+, and CD4+CD26+ and analyzed by fluorescence-activated cell sorter at the University of Tennessee Molecular Resource Center.

Preparation and handling of bovine CI. Bovine CI was prepared as previously described (19). Bovine fetuses from pregnant cows were obtained from a local slaughterhouse within 1 hour of death. The skins of 4 fetal calves were removed and maintained at 4°C throughout the preparation. The tissue was sliced into strips and processed through a household meat grinder, then homogenized in a Waring blender with ice chips. The homogenate was centrifuged (10,000g) for 30 minutes and reextracted twice with 1M NaCl (pH 7.6, with 0.05M Tris HCl) and twice with 0.1M acetic acid to remove some type III soluble collagen and much of the noncollagenous components. The final pellet (~500 gm) was suspended in 16 liters of 0.1M acetic acid, and the pH was adjusted to 2.8 with formic acid.

Type I collagen was solubilized by overnight (16 hours) digestion with 20 gm of pepsin (3X crystallized; Sigma, St. Louis, MO) at 4°C. The digest was centrifuged (10,000g for 30 minutes), and the insoluble pellet was discarded. Type I collagen in the supernatant was precipitated by addition of 5M NaCl solution to a final concentration of 0.8M. This was centrifuged as before, and the pellet was redissovled in 0.1M acetic acid. The pH was adjusted to 7.4 with 0.05M Tris and 10M NaOH to inactivate pepsin. Solid NaCl was added to a concentration of 1M, and the solution was centrifuged. The supernatant was collected, and the NaCl content was increased to 1.7M with 5M NaCl. This was centrifuged to remove contaminating CIII. The 1.7M NaCl supernatant was further adjusted to 2.5M NaCl, which precipitated the CI.

The CI pellet was collected by centrifugation and redissovled in 0.5M NaCl, 0.05M Tris, diluted to 0.2M NaCl with water, and 50 gm of DE-52 was added to create a slurry.

This was stirred overnight and centrifuged to remove any DEAE that had bound any remaining pepsin and contaminating glycosaminoglycans. The supernatant was dialyzed against 0.02M NaH2PO4 to precipitate CI. The pellet was redissovled in 0.01M acetic acid, dialyzed exhaustively against the same, and stored at −80°C until used.

The homogeneity of the CI was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which showed an α1(1)/α2(1) ratio of 2:1 with no contaminating type V or type III collagen. The α1(I) and α2(I) collagen chains were separated by carboxymethyl cellulose chromatography, and constituent α chains were digested with cyanogen bromide (19).

Frozen CI stock containers were allowed to thaw over 2–3 days at 4°C prior to dispensing into vials. Thawed collagen was centrifuged at 4°C at 12,000g to remove particulates. The collagen was diluted to a concentration of 50 μg/ml or 250 μg/ml with cold (4°C) 0.1M acetic acid. The diluted CI was filtered at 4°C through a glass fiber Acrodisc (Gelman Sciences, Ann Arbor, MI) and then a 0.45μ filter (Nalgene filter; Nalge, Rochester, NY) and aliquoted (2 ml) into sterile 2-ml screw-top polypropylene vials (Nalgene vials; Nalge). Vials were placed in plastic bags (35 vials/bag) and stored frozen at −20°C until given to the patients.

Statistical analysis. Cytokines produced by PBMC in response to culture with bovine CI, serum levels of sIL-2R, results of PFTs, and clinical variables were analyzed by Student’s paired t-test to determine whether significant changes occurred after 3, 6, 9, or 12 months of oral CI treatment, compared with pretreatment values. Correlations of the M-HAQ or the MRSS versus IL-10 or IFNγ were analyzed by Spearman’s correlation test.

RESULTS

Patient characteristics at study entry. Twenty-five patients with SSc were screened. Twenty-four exhibited production of IL-10 or IFNγ that was ≥2-fold higher than the levels in parallel cultures of the patients’ PBMC plus PBS but without α1(I) or α2(I). Four patients had complications of SSc or other medical diseases that disqualified them from the study. One patient was enrolled but withdrew from the study very early. The characteristics of the remaining 19 patients are shown in Table 1.

The patients were predominantly female, and the majority had late, diffuse disease. All patients satisfied the ACR preliminary criteria for the classification of SSc. Thirteen were white and 6 were African American. Only 3 patients had a disease duration of <2 years. Only 3 patients were currently taking D-penicillamine. The 5 patients taking nonsteroidal antiinflammatory drugs (NSAIDs) discontinued these during the last 6 months of CI treatment. Three patients took 5 or 10 mg/day of prednisone throughout the study period.

Side effects, withdrawals, and compliance. Seventeen patients were treated for 12 months. Two patients dropped out because of difficulty with transportation: one
very early (<1 month), the other after 6 months. One patient developed a foot drop of uncertain etiology and was removed from the study after 6 months of therapy. No other possible side effects were noted. There was 100% compliance by each patient until the time each dropped out of the study or the study was completed.

Induction of T cell tolerance to CI by administration of oral CI. The daily administration of bovine CI for 12 months was accompanied by significant reductions in IFNγ production by PBMC cultured with purified α1(I) and α2(I) chains of human CI as measured after 6 and 12 months of treatment (Figure 1A). IFNγ is a Th1 cytokine, and its reduced production by α1(I)- and α2(I)-stimulated PBMC suggests that oral tolerance to CI was effected. Quite surprisingly, IL-10 levels in the same PBMC culture supernatants were also significantly reduced after 3, 6, and 12 months of oral CI treatment (Figure 1B). The production of IFNγ and IL-10 by PBMC stimulated with PHA was not statistically different before or at 3, 6, or 12 months after oral CI treatment (results not shown).

After 12 months of oral CI treatment, there was a significant reduction (as determined by Student’s paired t-test) in the serum levels of sIL-2R (Figure 2).

T cell subsets measured by the following markers did not change after treatment with oral CI treatment: CD8+, CD4+, CD3+DR+, CD4+CD45 RA+, CD4+CD45 R0+, CD4+CD26+, CD8+CD45 RA+, and CD8+CD45 RO+ (results not shown).

**Table 1.** Characteristics of the patients taking oral type I collagen for 1–12 months*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
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<td>Sex</td>
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</tr>
<tr>
<td>Race</td>
<td>White 13</td>
</tr>
<tr>
<td>SSc type</td>
<td>Diffuse SSc 14</td>
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<tr>
<td>Age, mean ± SD years</td>
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<tr>
<td>Disease duration</td>
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<td>&lt;2 years’ duration</td>
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</tr>
<tr>
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<tr>
<td>NSAIDs</td>
<td>5</td>
</tr>
<tr>
<td>Prednisone</td>
<td>3</td>
</tr>
</tbody>
</table>

* Except as noted otherwise, values are the number of patients. SSc = systemic sclerosis; NSAIDs = nonsteroidal antiinflammatory drugs.

**Figure 1.** Production of A, interferon-γ (IFNγ) and B, interleukin-10 (IL-10) by peripheral blood mononuclear cells (PBMC) from patients with systemic sclerosis. PBMC were cultured with α1(I) and α2(I) chains before and after 3, 6, and 12 months of oral treatment with bovine type I collagen (CI; 500 μg/day). Harvested culture supernatants were analyzed for levels of IFNγ and IL-10 by commercial enzyme-linked immunosorbent assay, as described in Patients and Methods. Values are the mean and SEM. P values determined by Student’s paired t-test; NS = not significant.
**Improvement in clinical variables.** The M-HAQ difficulty in performing activities of daily living (ADL) scale and the MRSS were significantly improved after 6 and 12 months in this open-label study (Figures 3A and B). After 12 months of CI treatment, the M-HAQ ADL difficulty scale had improved 27%, from a baseline value of 0.66 ± 0.14 (mean ± SEM) to a value of 0.48 ± 0.14 ($P < 0.05$). The MRSS declined steadily, and after 12 months of CI treatment, had decreased by 23%, from a baseline value of 26.35 ± 2.35 to a value of 20.29 ± 2.53 ($P < 0.005$) (Figure 3B). In the patients with diffuse SSc, the MRSS decreased by 26.6% after 12 months of CI treatment, from a baseline value of 28.6 ± 2.5 to a value of 21.0 ± 2.7 ($P < 0.005$) (results not shown). There were no significant correlations between MRSS score or M-HAQ score and decreases in IL-10 or IFN-γ production by PBMC cultured with CI α chains after 12 months of CI treatment (results not shown).

Because of patient noncompliance and scheduling problems, only 11 of the 17 patients who finished the study had DLCO measurements and PFTs performed at 0 and 12 months. For these 11 patients, the mean DLCO, corrected for alveolar volume and hemoglobin, increased by 9.58% from 3.34 to 3.66 ml/minute/mm Hg ($P$...
DISCUSSION

In patients with limited and diffuse SSc, the administration of oral bovine CI for 1 month at a dosage of 100 μg/day and for 11 months at 500 μg/day resulted in significant reductions in IFNγ and IL-10 production by CI α-chain–stimulated PBMC. Levels of serum sIL-2R were also significantly reduced after induction of oral tolerance to CI. This reduction in sIL-2R, although small, suggests that overall T cell stimulation was reduced. To our knowledge, levels of sIL-2R in sera have not been reported in other studies of human oral tolerance or in animal models of oral tolerance. Therefore, the significance of this modest reduction in serum sIL-2R levels in the context of oral tolerance is not known at present. Taken together, these data indicate that oral tolerance to CI was effected by this treatment regimen.

The mechanism(s) by which this oral CI regimen induced these immune changes is not readily apparent. It is likely that IFNγ production by CI α-chain–stimulated PBMC is largely from CD4+ Th1 cells, but natural killer cells are also a potential source of this cytokine. The reduced production of IFNγ by PBMC CD4+ T cells could be due to 1 or a combination of the 3 mechanisms of oral tolerance induction (i.e., suppressive regulatory T cells, clonal anergy, or clonal deletion) (10–12).

The reduced IL-10 production by SSc CI α-chain–stimulated PBMC after oral CI treatment was unexpected, given that IL-10 has been reported to be up-regulated in peripheral lymphoid tissue or in target organs in autoimmune immune models after oral tolerance induction by low-dose antigen (20). However, in some circumstances, IL-10 can be produced by Th1 cells, and in humans, there is less rigidity to the Th1/Th2 paradigm originally described using clonal mouse T cells (20–22). In addition, IL-10 is produced by cells other than CD4+ T cells (23,24). Monocyte/macrophages are a major source of this cytokine (23,25). The reduced IL-10 production by CI α-chain–stimulated SSc PBMC after oral CI treatment may reflect overall decreased T cell responsiveness to the α chains, and therefore decreased stimulation to monocytes by IFNγ or other cytokines from T cells that up-regulate IL-10 production by monocytes. Finally, published studies of oral tolerance in animal models have not measured antigen-stimulated PBMC production of IL-10, or other cytokines elaborated by PBMC, before and after oral tolerance induction. The published studies of animal models of oral tolerance all measure cytokine expression in lymphoid tissue or target organs rather than peripheral blood.

NSAIDs are known to inhibit oral tolerance in animal models and may be a confounding factor in human oral tolerance (26–28). For this reason, we advised the 5 patients who had been taking NSAIDs for the first 6 months of the trial to discontinue them for the last 6 months of the trial, which they did.

Although there were significant improvements in the MRSS and M-HAQ scores, these findings should be viewed with caution, since this was an open-label study and these changes may reflect variations or spontaneous changes in the disease or a placebo effect. The DLCO values, while showing statistically significant improvement, are still just below the clinically significant cutoff of ≈10%. Clearly, a larger population of more homogeneous patients with diffuse SSc needs to be evaluated in a randomized, double-blind, placebo-controlled study before it can be categorically concluded that oral CI tolerance induction ameliorates the SSc disease process.

One could speculate that the mechanism by which oral CI might possibly ameliorate SSc could involve anergy and/or suppression depending on the
dose of CI given. Although the 500 µg/day dosage of CI induced oral tolerance, clearly other dosages need to be studied. The feeding of CI to SSc patients could anergize autoreactive cells and/or generate major histocompatibility complex class I– or class II–restricted regulatory T cells that sequester in involved tissues, where they release small amounts of immunosuppressive cytokines (IL-4, IL-10, TGFβ1) that down-regulate autoaggressive cells by the mechanism of antigen-driven bystander suppression. By antigen-driven bystander suppression, these CI-specific T cells could down-regulate T cell interactions with other antigens (29), as has been demonstrated in autoimmune animal models in which oral tolerance has been induced by oral administration of antigens from organs that are the target of attack.

These animal models provide a theoretical basis for predicting that in SSc patients, CI, although it may not be an initiating antigen of SSc or even be involved in its pathogenesis, when given as an oral tolerogen, may well suppress T cell–mediated fibrogenesis by suppressing activated T cells. If activated CD4+ T cells present in the tissues of SSc patients could be down-regulated, then with time, the fibrogenic phenotype of SSc fibroblasts might revert to normal. It is known that after serial passage of SSc fibroblasts in vitro for several generations, they regain a more normal phenotype with regard to matrix synthesis (30). Also, patients with longstanding SSc tend to have less skin thickening and collagen deposition than they had in earlier stages of their disease.

While IL-4 and TGFβ at high concentrations (~50 ng/ml and 5 ng/ml, respectively) can up-regulate collagen synthesis by cultured fibroblasts in vitro, lower concentrations (e.g., TGFβ1 at 1,000 times less) are capable of modulating immune cells (30–34). The fact that oral CI treatment did not increase skin or lung fibrosis suggests that if GALT-derived regulatory T cells producing these cytokines were generated by oral CI treatment, the levels of these cytokines are likely to be lower than is required to trigger collagen synthesis by fibroblasts.

Oral CI administration appears to be safe in SSc patients. Its efficacy needs to be assessed by a larger placebo-controlled, double-blind trial.

ACKNOWLEDGMENTS

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REFERENCES


10. Friedman A, Weiner HL. Induction of anergy and/or active suppression in oral tolerance is determined by frequency of feeding and antigen dosage [abstract]. J Immunol 1993;150:4A.


Errata

In the article by García-Porrua et al published in the March 2000 issue of *Arthritis & Rheumatism* (pp 584–592), there was an error in the second full sentence in the left column on page 589. The sentence should have read, “Six of 39 patients diagnosed as having idiopathic EN (15.4%) had 1 or more predictive factors for secondary EN, and 64 of 67 patients diagnosed as having secondary EN (95.5%) had 1 or more predictive factors for secondary EN” [emphasis added]. This is also how the first sentence of the first footnote in Table 4 should have read.

In the article by van der Heijden et al in the March 2000 issue (pp 593–598), the reference cited at the end of the first sentence in the second paragraph of Patients and Methods (page 594) should have been reference 11, rather than reference 4. The reference cited at the end of line 12 in the second paragraph of the Discussion (page 597) should also have been reference 11, rather than reference 8.

We regret the errors.