Immune response after collagen injection for laryngeal rehabilitation: development of an enzyme-linked immunosorbent assay

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Summary. The appearance of circulating antibodies directed against bovine collagen has been observed in dermatology after collagen injections with or without clinical reactions. Knowing the serologically positive patients among the clinically negatives ones is important if a second collagen injection should be planned. In these cases, skin and blood tests have to be carefully checked to confirm reactivity. In connection with this, we have developed an enzyme-linked immunosorbent assay (ELISA) for determining the response to GAX collagen injections. Such injections induce a very low rate of anti-collagen antibodies. However, without having recourse to a positive control permitting statistical analysis of data, no conclusions can be made about significant positivity. In the present investigation of 12 patients following GAX collagen injections into the larynx, 1 was found to be borderline and 1 positive (17%). The positive control was made up with 20 ng/ml anti-type III collagen antibodies, which was purified and tested against type III bovine and human collagen. The present study showed that the systematic detection of antinuclear or anti-DNA antibodies after GAX collagen injection is not useful. However, the anti-collagen positivity of two control samples from 3 patients already positive for antinuclear antibodies demonstrates that bovine collagen injections are contraindicated in auto-immune disease.

Key words: Collagen injection – Immune response – Laryngeal rehabilitation

Introduction

Injectable collagen has been widely used in cosmetic surgery for the correction of dermal contour deformities for more than 10 years [4]. Since 1984, it has also been used on more than 300 patients for vocal and laryngeal rehabilitation [5, 10]. The first type available was ZCI collagen (Zyderm, Collagen Corp., Palo Alto, Calif., USA). Since 1986, it has been replaced by GAX collagen (Phonagel, Collagen Corp.) for its laryngeal application [8]. Both substances are highly purified, solubilized bovine collagen, which is well known for its low immunogenicity [1-4]. Nevertheless, prior to treatment with ZCI or GAX, all patients are given an intradermal skin test with 0.1 ml ZCI to detect potential sensitivity to this foreign implant material. If there is no reaction (e.g. edema, induration, erythema, pruritus and/or tenderness or pain) during the ensuing month, treatment can be initiated. Systemic manifestations (rashes, myalgias and pruritus) have occasionally accompanied some of the test site reactions to ZCI. Most of these reactions last only a few days, but some have persisted for several months before they spontaneously resolved.

There is a 3.0% positive reaction rate to the skin test [4] and a number of adverse reactions have also occurred following treatment with ZCI. Some of these have been characterized clinically by the development of edema, erythema, induration and/or urticaria at the implantation sites, and have been considered to be allergic in nature. The incidence for these adverse treatment reactions has been less than 1–5% [4, 5, 8, 10, 11]. To date, there has been relatively little concern about such low reactions as redness, firmness or slight swelling in the larynx, compared to the same reactions in cosmetic surgery. Since there has also been an absence of an immune reaction to collagen in our series of patients [9, 10], and only two positive skin tests to ZCI collagen have been reported by Ford et al. [6], we have shortened our tolerance test observation period to only 10 days. As a result, we have been able to treat more rapidly problems resulting from laryngeal or glottic insufficiency (e.g., poor deglutition after subtotal laryngectomy) [9]. However, there is still concern voiced clinically about the possibility of collagen injections causing not only a local immune response but also a humoral one.
Positive correlations have been noted between the appearance of adverse clinical reactions to bovine collagen implants and the appearance of circulating antibodies directed against bovine collagen [2, 3, 11]. Serologic reactions have occurred in some patients treated with bovine collagen implants but without adverse clinical reactions and a positive serology has also been found in a low percentage of untreated individuals. That immune responsiveness to collagen is genetically determined and in man may be related to the human leukocyte antigen (HLA) type of the patient [12]. As such, we wished to carry out our own serologic tests so as to be able to detect any serologically positive patients among all our clinically negative ones. Knowing whether or not a patient was positive serologically is important if a second collagen injection is planned. We now report our findings.

Patients and methods

Two groups of patients were tested successively. The first group (group A) consisted of 19 patients and was composed of 14 males and 5 males. The patients ranged in age from 14 to 74 years, with a mean age of 52. The second group (group B) contained 12 patients (2 females, 10 males). Their ages ranged from 42 to 67 years, with a mean age of 54. All of the patients were injected with GAX collagen for vocal or laryngeal rehabilitation after giving negative skin tests for reactivity. Only one procedure was needed for all of the patients. The volume of the implant received per patient varied from 0.5 ml in cases with paramedian vocal fold paralysis to 3 ml or more in cases with subtotal laryngectomy. No patient had a history of auto-immune disease. Blood was taken from all patients at their first follow-up medical examinations, about 15 days after injection.

With the intention of controlling our test for cases with possible immunologic disease, blood samples were also analyzed from 3 patients with positive antinuclear antibodies (FAN) and from 1 patient with anti-desoxyribonucleic acid (DNA) antibodies.

Serum samples. The samples were collected by routine veni-puncture under aseptic conditions and placed into heparin-free tubes. The blood was allowed to clot 30 min at 37°C and the serum was separated by centrifugation. The serum samples were aliquoted and stored at −70°C until use.

Enzyme-linked immunosorbent assay. All sera were tested by enzyme-linked immunosorbent assay (ELISA). Type III collagen (Sigma, St. Louis, Mo., USA), derived from calf skin, was suspended in acetic acid 0.1N, on a basis to 0.1 mg collagen/ml. This collagen was denatured for 30 min at 60°C. The solution so obtained was diluted 1:20 with 0.1 M carbonate buffer, pH 9.6. A plastic microtiter plate (Nunc flat – bottomed – quality 1: Roskilde, Denmark) was then coated with the diluted collagen solution. This was accomplished by adding 100 ml of the freshly diluted solution to each well of the microtiter plate and incubating the plate for 2 h and 37°C. Every sample was diluted 1:10 in phosphate-buffered saline (PBS) at pH 7.3 containing 0.02% magnesium chloride hexahydrated and 0.05% polysorbate (tween 20).

Four group B studies, a positive control was made up with 20 ng/ml anti-type III collagen antibodies, purified and tested for type III bovine and human collagen (Sera-lab no. SBA 1330-01; Crawley Down, UK). Three off-study FAN-positive samples and one DNA-positive sample were also prepared.

Each microtiter plate was washed three times with PBS-tween. Then, 200 ml of each diluted sample and controls (group B) were added to the wells. This was allowed to incubate for 30 min at 37°C. The microtiter plate was again washed three times with PBS-tween. One hundred microliters of peroxidase-conjugated rabbit anti-human IgG (gamma) (Dako no. p214; Glostrup, Denmark) diluted 1:100 with PBS-tween with 25% heat inactivated fetal calf serum was added. This was again incubated for 30 min at 37°C. The microtiter plate is then washed three times more with PBS-tween, after which 200 ml working solution was added. Stock solution contained 0.1 g of 2,2'-azino-bis (3-ethylbenzthiazoline sulfonic acid diammonium salt (Sigma no. A-1888), 1.3 g critic acid and 1.55 g disodium hydrogenophosphate dihydrated per 100 ml distilled water. Working solution was made by mixing 5 ml of 33% volume hydrogen peroxide to 20 ml of 1/20 diluted working solution. The coloring development was observed for the following 15 min. Absorbance measurements at 405 nm (A405) were obtained.

Detection of antinuclear antibodies (FAN) anti-DNA antibodies. In order to detect patients with subclinical auto-immune disease among those possibly positive for ELISA, all group A patients were tested for FAN and anti-DNA antibodies.

Antinuclear antibodies (FAN). A commercially available semi-quantitative technique of antinuclear antibodies detection by immunofluorescence (Immuno-Concepts, Sacramento, Calif.) was used. The first layer fixation of serum antibodies on the HEp-2 cell nucleus during mitoses was affected. Demonstration of fixation was obtained with goat anti-human immunoglobulin G (heavy and light chains) combined with fluorescein isothiocyanate (FITC).

Anti-DNA antibodies. A commercially available semi-quantitative technique for the detection of anti-DNA antibodies by immunofluorescence (Immuno-Concepts) was used. Antibody fixation was effected on kinetoplasts or on kinetoplasts and Crithidia luciliae nuclei with high DNA content. Fixation was shown with goat anti-human immunoglobulin G (heavy and light chains) combined with FITC.

Computed data. For the group B patients, data ratios were determined from the A405 values for each patient. Each ratio was determined by dividing the sample value by the mean values of all patients' samples.

Results

Group A patients

All absorbances at 405 nm (A405) measurements were very low and were considered to be negative for collagen antibodies as well as for FAN and anti-DNA antibodies.

Group B patients

The results are shown in Table 1. The serum sample optical density mean value was 0.1715 ± 0.0392. For 2 SDs, the upper limit value was 0.2498 and the lower limit value 0.0930. For 3 SDs, the upper limit value was 0.2890 and the lower limit value 0.0538. The normal ratio superior limit was 1.457 [upper limit value (+ 2 SDs)/mean value] for 2 SDs, and 1.686 for 3 SDs [upper limit value (+ 3 SDs)/mean value].

Discussion

GAX collagen injection induces a very low incidence of anti-collagen antibodies detectable by the ELISA test. Without having recourse to a positive control permitting statistical analysis of the data, it was not possible to dem-