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Milos Chvapil and Thomas A. Chvapil

Department of Surgery,
University of Arizona Health Sciences Center

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TEL. 東京（664）7750（代表）

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Milos Chvapil and Thomas A. Chvapil

Department of Surgery,
University of Arizona Health Sciences Center

Tucson, AZ 85724, U.S.A.

ABSTRACT

A total of thirty New Zealand white male rabbits were injected intradermally using multiple dorsal sites with collagen fleece (Novacol) or fibrous form (Avitene), either plain or in an emulsion with Freunds adjuvant. Identical booster injections of the same dose as the initial challenge were given 22 days later. All animals were bled at 28 days after the booster injections. Using the sensitive ELISA method, it is concluded that collagen fleece did not induce antibody formation.

INTRODUCTION

The antigenicity of implantable biomedical products based on collagen protein has been rightfully a continuing concern of involved researchers as well as regulating agencies. The voluminous literature on collagen antigenicity could be summarized as follows: the pure collagen molecule is a rather weak antigen, approximately 50 times weaker than globular proteins, such as serum bovine albumin. Further reduction of the antigenicity can be achieved by cleaving the nonhelical peptides at N- and C-terminals of a collagen polypeptide chain or by introducing artificially new intra or intermolecular cross-links in the collagen triple-helical molecules. Three antigenic determinants have been identified in collagen:

1) The major antigenicity is in the telopeptides region. As indicated, telopeptides can be cleaved by pepsin digestion as in the case with collagen-Zyderm, manufactured by Collagen Corporation (CA).
2) The other antigenic determinant resides in a cyanogen bromide cleaved peptide 5.
3) The third determinant is associated with the helicity-crystallinity of collagen[1-8].

Collagen-based products used for topical hemostasis consist mostly of noncross-linked

collagen, as additional cross-linking diminishes the hemostatic effectiveness[4]. Insoluble collagen must be first solubilized by macrophages and only then serves as an antigen. Hemostatic collagen is solubilized (biodegraded) at various rates when in contact with tissues. Recently, we showed[5], that subcutaneously implanted collagen fleece is resorbed within 4 weeks while fibrous form is biodegraded at a pace several times slower. This aspect may definitely affect the antigenicity of the product.

MATERIALS AND METHODS

1 Materials

The Hemostatic Collagen Fleece (collagen fleece, Novacol, Bioplex Corp., a subsidiary of Datascope Corp., NJ, U.S.A.) used in this study was isolated from bovine achilles tendons by a combined chemical and mechanical patented procedure[6]. Microfibrillar Collagen Hemostat (fibrous form, Avitene) was supplied by Avicon Incorporated, Fort Worth, TX. This product was manufactured by a procedure described by Battista and Cruz[7].

2 Animals, grouping and treatments

A total of thirty New Zealand white male rabbits, body weight 1.5–2.0 kg were randomly distributed into five groups containing six rabbits each.

Group	Treatment
1	Sham control receiving injection of 0.05% acetic acid
2	Injected collagen fleece alone (Novacol, Datascope Corp, NJ)
3	Injected fibrous form alone (Avitene, Avicon Corp, TX)
4	Injected collagen fleece and Freunds adjuvant 1 : 1
5	Injected fibrous form and Freunds adjuvant 1 : 1

Each group was injected intradermally on the back with the respective materials. Each dose was divided into 10–14 individual injections. Identical booster injections of the same dose as the initial shots, were given 22 days later. All animals were bled via the ear artery at 28 days after the booster injections; all sera were frozen at −70°C and used for antibodies assay within the next 10 days.

3 Materials injected

a) Sham: 0.05% acetic acid, given at a dose of 1.2 m*l*/animal.

b) Collagen fleece was prepared by disintegrating the fleece; this was facilitated by soaking it in 70% alcohol and subsequent freezing and powderizing it in liquid nitrogen. Dry weight of the produced powder was established and the material partially suspended/dissolved in 0.05% acetic acid, at a concentration of 10.75 mg/m*l*. Each animal was injected with 1.2 m*l* of this solution initially and at 22 days.

c) Fibrous form solution was prepared by dissolving sterile powder in 0.05% acetic acid;

final concentration was 11.43 mg/m*l*. Each animal in this group was injected with 1.1 m*l* initially and at 22 days. The actual concentration of either injected product was checked by direct determination of hydroxyproline (Hyp) in an acid hydrolyzed sample[8]. A factor of 7.34 was used to convert the values of Hyp to collagen.

d) Collagen fleece with Freunds complete adjuvant: prepared in the same manner as the collagen fleece; however, equal parts of collagen fleece solution and Freunds complete adjuvant were mixed (emulsified) together.

e) Fibrous form with Freunds complete adjuvant: equal parts of fibrous form solution and Freunds mixed (emulsified) together.

4 Serum antibody determination by the ELISA method

The ELISA procedure for this specific application followed the method of Rennard, *et al*[9]. In order to establish the feasibility of using the ELISA method, a standard curve of known antibody (Ab) concentration (Rabbit anti-bovine collagen IgG, obtained from Dr. Steffen Gay, University of Alabama, Birmingham, AL) had to be obtained. From the titration curves of different Ab dilutions and antigen concentrations, the optimal conditions were selected, giving the greatest separation of optical densities, as follows:

Coating of plates: Antigens were diluted from acetic acid solutions into 20 mM carbonate buffer, pH 9.6, containing 0.02% sodium azide and allowed to adsorb to the microtiter wells at 4°C. Concentration of collagen was 200 ng/well.

Serum Ab dilutions: After coating the plates, serum with antibodies was diluted with phosphate-buffered saline (20 mM sodium phosphate, 0.15 M sodium chloride) with 0.05% Tween 20 and a constant amount of antibodies was added to antigen-coated plate. Antibody dilutions were 1 : 10 for plain collagen fleece or fibrous form and sham and 1 : 50 for collagen fleece or fibrous from with Freunds adjuvant and shams. All analyses were done in triplicates.

ELISA Assay: The plates were incubated for two hours at room temperature with shaking, then washed with the carbonate buffer. Anti-rabbit IgG alkaline phosphatase conjugate (Sigma # A-802f) was added to each well and incubated for one hour with shaking. The enzyme was used at concentration of 1 : 1000. After washing the plates with the carbonate buffer, enzyme substrate (*p*-nitrophenyl phosphate, disodium, Sigma # 104-D) was added and incubated for 15 min. The substrate was used at a concentration of 1 : 10. The enzyme reactions were stopped with 3N NaOH. All readings of the colors developed were made on Dynatech Microelisa plate reader and Titertek Multiskan reader (Flow Laboratories, Inc.) at 405 nm.

RESULTS

The results of the antigenicity of plain collagen fleece (Novacol) and fibrous form (Avitene) for individual rabbits are presented in **Table 1**. Analysis of the data of **Table 1** indicates that collagen fleece induced substantially lower production of antibodies as documented

Table 1 Optical densities of sera from rabbits injected with collagen hemostats and shams, as processed by ELISA test

Rabbit #	Material Tested (Antigen)	Serum Dilution	Fibrous form Coated Plates (O.D. ± S.D.)	Collagen fleece Coated Plates (O.D. ± S.D.)
1 A	Collagen fleece	1 : 10	0. 025 ± 0. 014	0. 061 ± 0. 006
2 A	Collagen fleece	1 : 10	0. 021 ± 0. 018	0. 358 ± 0. 013*
3 A	Collagen fleece	1 : 10	0. 089 ± 0. 016	0. 075 ± 0. 005
4 A	Collagen fleece	1 : 10	0. 123 ± 0. 017	0. 155 ± 0. 020
5 A	Collagen fleece	1 : 10	0. 133 ± 0. 016	0. 037 ± 0. 005
6 A	Collagen fleece	1 : 10	0. 091 ± 0. 017	0. 086 ± 0. 011
1 B	Fibrous form	1 : 10	0. 468 ± 0. 019	0. 108 ± 0. 040
2 B	Fibrous form	1 : 10	0. 683 ± 0. 026	0. 271 ± 0. 060
3 B	Fibrous form	1 : 10	0. 231 ± 0. 023	—
4 B	Fibrous form	1 : 10	0. 395 ± 0. 028	0. 171 ± 0. 050
5 B	Fibrous form	1 : 10	0. 688 ± 0. 033	0. 179 ± 0. 040
6 B	Fibrous form	1 : 10	0. 754 ± 0. 037	0. 283 ± 0. 040
1	Sham	1 : 10	0. 013 ± 0. 010	0. 052 ± 0. 010
2	Sham	1 : 10	0. 030 ± 0. 019	0. 035 ± 0. 010
3	Sham	1 : 10	0. 027 ± 0. 021	0
4	Sham	1 : 10	0. 186 ± 0. 022	0
5	Sham	1 : 10	0. 031 ± 0. 019	0
1 A	Collagen fleece	1 : 50	0. 023 ± 0. 015	0
3 A	Collagen fleece	1 : 50	0. 019 ± 0. 101	0
4 A	Collagen fleece	1 : 50	0. 033 ± 0. 014	0
1 B	Fibrous form	1 : 50	0. 151 ± 0. 017	0
2 B	Fibrous form	1 : 50	0. 382 ± 0. 015	0
3 B	Fibrous form	1 : 50	0. 134 ± 0. 016	0

O.D. readings were corrected for "blank" background, measured in complete system without antigen.
* : Hemolysed

by lower readings at 405 nm in plates coated either with collagen fleece or fibrous form antigen. Both tested collagen products possibly consisted in pure type I collagen of bovine origin, which should result in excellent crossreactivity between the tested collagens. When the antibody production was enhanced by mixing either antigen with Freunds adjuvant (see **Table 2**), the formation of antibodies against either antigen was the same.

The data for individual rabbits were statistically treated and the resulting summary, **Tables 3** and **4**, show that:

1) Fibrous form is considerably more antigenic than collagen fleece. This difference is highly statistically significant ($p<0.01$).

Table 2 Optical densities of sera from rabbits injected with collagen hemostats mixed with Freunds adjuvant, and shams, as processed by ELISA test

Rabbit #	Material Tested (Antigen)	Serum Dilution	Fibrous form Coated Plates (O.D.±S.D.)	Collagen fleece Coated Plates (O.D.±S.D.)
1 C	Collagen fleece with Freunds adjuvant	1 : 50	0.622±0.016	0.209±0.04
2 C	Collagen fleece with Freunds adjuvant	1 : 50	0.749±0.028	0.519±0.04
3 C	Collagen fleece with Freunds adjuvant	1 : 50	0.703±0.025	0.289±0.05
4 C	Collagen fleece with Freunds adjuvant	1 : 50	0.331±0.018	0.051±0.02
5 C	Collagen fleece with Freunds adjuvant	1 : 50	0.923±0.028	0.345±0.04
6 C	Collagen fleece with Freunds adjuvant	1 : 50	0.110±0.018	0.093±0.02
1 D	Fibrous form with Freunds adjuvant	1 : 50	0.764±0.022	0.099±0.04
2 D	Fibrous form with Freunds adjuvant	1 : 50	0.228±0.011	0
3 D	Fibrous form with Freunds adjuvant	1 : 50	0.654±0.015	0
4 D	Fibrous form with Freunds adjuvant	1 : 50	0.464±0.017	0
5 D	Fibrous form with Freunds adjuvant	1 : 50	0.266±0.020	0
6 D	Fibrous form with Freunds adjuvant	1 : 50	0.310±0.014	0
1	Sham	1 : 50	0.027±0.011	0
2	Sham	1 : 50	0.044±0.023	0
3	Sham	1 : 50	0.073±0.024	0
4	Sham	1 : 50	0.286±0.042	0
5	Sham	1 : 50	0.085±0.039	0
Blank				0.095±0.070
1 C	Collagen fleece with Freunds adjuvant	1 : 100	0.466±0.018	0.090±0.050
2 C	Collagen fleece with Freunds adjuvant	1 : 100	0.620±0.025	0.394±0.060
3 C	Collagen fleece with Freunds adjuvant	1 : 100	0.577±0.033	0.312±0.040
1 D	Fibrous form with Freunds adjuvant	1 : 100	0.590±0.033	0
2 D	Fibrous form with Freunds adjuvant	1 : 100	0.222±0.053	0
3 D	Fibrous form with Freunds adjuvant	1 : 100	0.615±0.066	0

Table 3 Antigenicity of collagen hemostats

Materials Tested	N	O.D. ± S.D.	Significance
Fibrous form	5	0.537 ± 0.210	a
Collagen fleece	6	0.092 ± 0.040	b
Sham control	5	0.057 ± 0.070	b
Blank	6	0.023 ± 0.026	b

N refers to number of rabbits tested. Significance was calculated by Duncan's multiple variability range test. Different letters indicate statistically significant difference of values at $p<0.01$.

Table 4 Antigenicity of collagen hemostats injected with Freunds adjuvant

Materials Tested	N	O.D. ± S.D.	Significance
Fibrous form	5	0.448 ± 0.22	a
Collagen fleece	6	0.573 ± 0.29	a
Sham Control	5	0.103 ± 0.105	b
Blank	6	0.095 ± 0.007	b

N refers to number of rabbits tested. Significance was calculated by Duncan's multiple variability range test. Different letters indicate statistically significant difference of values at $p<0.05$.

2) There is no statistical difference between the collagen fleece and the sham control treated rabbits. Collagen fleece did not produce antibodies under the used experimental conditions.

3) When either fibrous form or collagen fleece were injected into rabbits together with Freunds adjuvant, both collagen preparations showed the same antibody formation capacity. No statistical difference exists between the two materials tested. Note that the sham treatment was done without Freunds adjuvant, which alone theoretically should not alter the observed immune response to our antigen coating.

The fibrous form collagen coated plates (200 ng/well) were superior to those of collagen fleece (200 ng/well). Even though we selected the fibrous form coated plates for the final summary presentation in **Tables** 3 and 4, all tests were run concurrently on collagen fleece collagen coated plates. The trend is the same, even though the differences between groups are not as striking as on the fibrous form coated plates.

As shown in **Table** 5, collagen fleece is more than five times less antigenic than fibrous form, if a comparison of relative antigenic response is related to fibrous form. Taking into consideration the above value of sham and blank it follows that collagen fleece did not induce any formation of antibodies.

Table 5 Relative antigenicity of collagen hemostats

Material Tested	% Antigenicity
Fibrous form	100±39
Collagen fleece	17±7
Sham control	11±13
Blanks	4±5

Fibrous form was considered as 100% antigenic.
Other groups are compared to fibrous form.

DISCUSSION

Several studies demonstrated[10,11] that the major antigenic determinant of the collagen molecule is located in both N- and C-terminal regions (telopeptides) of the molecule. One way to minimize the antigenicity of collagen is to cleave the telopeptides by pepsin; another is to reduce the *in vivo* solubility and rate of degradation of the implanted collagen product. In this case, it is assumed that slowly dissolving subthreshold amounts of collagen as an antigen will not induce antibody formation.

During several years of research with tissue implants of highly cross-linked collagen sponges, we have never observed any morphological or biological evidence of immunological reaction to the implant[1]. As indicated by Stenzel *et al*[12], antibodies to collagen have not been detected in patients' sera following implantation of cross-linked collagen biomaterials.

Numerous experimental evidence indicates low antigenicity of collagenous protein when compared with globular proteins[1,2]. Another set of data indicates no antigenicity of insoluble collagen[14].

The finding of Michaeli and Epstein[18] that denatured collagen has higher antigenicity strongly indicates the importance of the use of native collagen for medical applications.

Collagen forming the Hemostatic Collagen Fleece (collagen fleece, Novacol) structure consists of native collagen fibrils. It is assumed that in an average surgical procedure no more than 1 g of the material will be used. Although it is customarily recommended that surgeons remove the hemostatic material from the bleeding site after obtaining hemostatis, some surgeons prefer to leave the collagenous hemostat in the body. Resorption studies indicate that when collagen fleece is left *in situ*, the material is resorbed within 3-4 weeks. Faster solubilization and biodegradation of collagen fleece (Novacol) over fibrous form (Avitene)[5] should theoretically favor more antigenicity of collagen fleece, which is in conflict with results of this study. Thus, the other difference between collagen fleece and fibrous form collagen seems to be the degree of purity: small amounts of bovine serum-albumin-like protein (0.05-0.3%) was found intercalated in fibrous form[10]. This may explain our finding that complete amino acid composition of either product shows that both proteins are very pure collagens, still fibrous form contains 4.5 tyrosine residues/1000 amino acid residues, while collagen fleece had only 1.8 tyrosine/1000 residues. There was also higher phenyl-

alanine content in fibrous form when compared to collagen fleece (14.0 residues vs 11.7).

Based on the results of this study, we can conclude that collagen fleece did not induce antibody formation in the rabbit whereas fibrous form did induce antibody formation.

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