0.78 mg of colloid. In the thyroids after 30 days of methimazole treatment, the mean wet thyroid weight increased to 20.53 mg, but it is assumed that they lost 3.30 mg of colloid present in the control glands. This difference of 17.23 mg represents the increase in weight due to cell multiplication and cell size. The increase in cell multiplication measured by the increase of 64.14% in DNA multiplied by the difference in wet weight (17.23 mg) indicated an increase of 11.05 mg due to cell multiplication. The increase in weight due to cell multiplication is therefore 54% and to cell size 46%. These values do not take into consideration any alteration in the stroma.

Summary. Mature female rats were injected with 400 μg/100 g b.w. of methimazole/day for 20 and 30 days. In 20 days, the mean thyroid gland weight increased from 9.73 mg for the controls to 16.04 mg, an increase of 65% and after 30 days to 20.53 mg, an increase of 111%. To determine to what extent the increase in thyroid gland weight was due to cell multiplication and to an increase in cell size, the total DNA/gland and the DNA/mg DFFT was determined. After 20 days the DNA/gland increased 52% and after 30 days, 64%, whereas the DNA/mg DFFT remained constant in the 3 groups. It has been calculated that the increase in thyroid gland weight after 30 days of goitrogen treatment represents a 54% increase in cell growth (multiplication) and a 46% increase in cell size.

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Acceleration of Wound Healing with Heterologous Cartilage. Immunological Considerations.* (31542)

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Investigation of the process of wound healing has long been one of the prime interests in surgical laboratories. The most interesting facet of this effort has always been the hope of accelerating this biological process so as to shorten the healing time. Many substances are capable of producing relevant phenomena *in vitro*, but they do not increase the tensile strength of experimental wounds in the slightest(1). Only one substance has been demonstrated to produce a consistent acceleration in wound healing and this material is cartilage(2,3,4,5,6,7). Extracts of the material will produce an accelerator effect(8) as will local application of the material. These biological effects have been demonstrated with cartilage from a variety of species in wounds in a number of different animals(9). Furthermore, recent work from this laboratory demonstrates conclusively that cartilage preparations also accelerate human wound healing(10).

As the cartilage preparations have been of heterologous origin (cow, shark) and hence contain foreign substances, the problem of the antigenicity of such compounds must necessarily be considered.

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Previous work in this area has been performed in relationship to the use of bovine cartilage in reconstructive surgery. Here, the attention was directed towards the delayed hypersensitivity reaction. Use of heterologous cartilage in human reconstructive surgery has been attempted by Stout(11), Gillies and Kristensen(12), and also by Gibson and Davis(13). The last work was the most extensive and reported that of twelve bovine cartilage implants, all but two had been resorbed within two years. Part of their work to investigate the delayed hypersensitivity involved the insertion of small cubes of cartilage into the soft tissues. On initial exposure the host showed no reaction, but the second exposure incurred an inflammatory reaction characterized by lymphocytes and foreign body giant cells around the cartilage.

The authors concluded that immune mechanisms were involved on the basis that the response to the cartilage was increased with increasing time of exposure. The tissue reaction was also found to be directly related to the amount of surface area of the graft. In sum, the use of heterologous cartilage was found unsatisfactory for reconstructive work because of the eventual resorption of the graft.

In the present use of bovine cartilage, the morphological structure is of no import since the material is being used for pharmacological rather than anatomic properties. In keeping with this use, it is important to investigate the possible production of immediate hypersensitivity which might result in anaphylaxis or in a serum sickness type reaction.

To evaluate the antigenic potential of the material, rabbits were exposed to the cartilage and assayed for precipitating antibodies. Human subjects with previous exposure to bovine cartilage were assayed for skin sensitizing activity as well as precipitating antibodies.

Method. Cartilage: The cartilage used is of bovine tracheal origin.§ Upon removal from the animal the soft tissues are removed by dissection and by acid pepsin digestion. The cartilage is then ground until the powder passes a #325 mesh screen (44 μ).

§ Obtained from Leslie Balassa Associates, Ltd., Scarborough, N. Y. under FDA Ind #2130.

Rabbit sensitization: The animals used were New Zealand male white rabbits of approximately 4000 g weight.

A. In the first set of rabbits, subcutaneous implantation of cartilage pellets was attempted as the sensitization procedure. With sterile technique, a skin incision 3 cm long was made through the skin on the anterior thigh of each rabbit. By means of a curved hemostat, 6 subcutaneous tunnels were made radiating from the incision and a cartilage pellet of 75 mg was placed at the end of each tunnel. The incision was closed with 3 vertical mattress sutures of 000 silk which were removed after one week. Four weeks later blood was drawn from the ear veins of both rabbits.

B. In the second set of rabbits, the cartilage preparation used for sensitization consisted of a normal saline extract of a compound which had previously demonstrated activity in accelerating wound healing in rats (lot A). The material was emulsified with complete Freund's adjuvant so as to contain 12.5 mg of cartilage extract per ml. 0.6 ml of this material was injected into the footpads of 3 rabbits. The initial injection was followed 3 and 4 weeks later by 1.0 ml of the same material injected intramuscularly. Two weeks thereafter, blood was obtained from an ear vein.

Immunological technique: The Ouchterlony gel diffusion technique(14) was used for the demonstration of precipitating antibodies.

Subjects: Four groups of subjects were used. Group I consisted of individuals who had received no exposure to the cartilage. Group II were laboratory workers who had been exposed to the cartilage by means of inhalation for varying periods of time as long as 8 years. Group III received topical application of the cartilage preparation on one of 2 paired wounds which were excised after a period of 7 to 14 days. Group IV received topical application of the cartilage on a wound during surgery without later excision of the wound. These individuals were tested 2 months to 18 months later.

Intradermal testing: Intradermal tests of the "wheal and erythema" type were performed by the injection of 0.02 ml of material

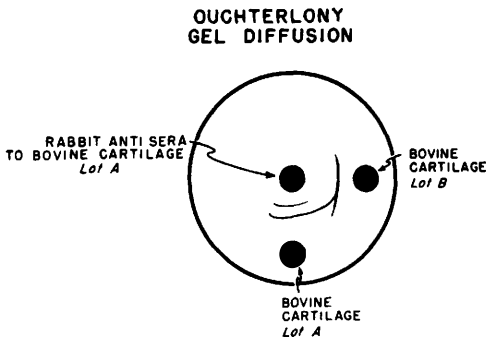

FIG. 1. Ouchterlony gel diffusion demonstrating precipitating rabbit antibodies to bovine cartilage preparations. Rabbit sensitized with bovine cartilage Lot A (active) emulsified with complete Freund's adjuvant. Bovine cartilage Lot B is also active in stimulating wound healing.

using a one ml syringe fitted with a #26 needle. These tests were then interpreted according to the criteria of Sherman and Kessler (15). Cartilage extract in normal saline was rendered sterile for testing with the use of a Millipore® Filter (Millipore Filter Corp., Bedford, Mass.). Final concentration for intradermal testing was 2.5 mg of cartilage per ml, which contained 10 g of protein nitrogen per ml. Other antigens used for intradermal testing were ragweed extract, bovine serum, and extracts of bovine milk, muscle and epithelium (Institute of Allergy, Roosevelt Hospital, New York). All subjects were evaluated for the presence of other clinical allergies.

Additional intradermal tests were performed in one subject with sterile aqueous solution of bovine hemoglobin, 2% (Colorado Serum Co., Denver, Col.) chondroitin sulfate, 5 mg per ml, N acetyl glucosamine, 25 mg per ml, galactosamine, 25 mg per ml, and pepsin, 0.05 mg per ml (all from Nutritional Biochemicals Corp., Cleveland, Ohio).

Data: The serum of rabbits receiving pellet implantation showed no precipitating antibodies to the saline extract of bovine cartilage or the other bovine antigens tested. The serum of rabbits immunized with the saline extract of bovine cartilage, lot A, emulsified with complete Freund's adjuvant showed at least two distinct precipitin lines against the cartilage extract which had been used for the immunization. A saline extract of another lot of bovine cartilage (lot B) also showed at least one definite precipitin line which showed incomplete antigenic identity with the initial material. These results are presented in Fig. 1.

Fig. 2 shows the results of Ouchterlony diffusion of the rabbit antisera against 2 different lots of cartilage preparation. Neither of these were used for the original immunizations of the rabbit. Lot B was active in the acceleration of wound healing while Lot C was inactive. The photograph demonstrates a single precipitin line of identity for the interaction of the rabbit antiserum against these 2 cartilage preparations. No precipitin reaction is demonstrable against the other materials tested, *i.e.,* human cartilage extract, chondroitin sulfate, bovine serum, bovine hemoglobin, and bovine pepsin.

Five normal control subjects (Group I) were skin tested with bovine cartilage extract (Lot A), 2.5 mg/ml, and the material at this concentration was found to be non-irritating. A similar preparation at a concentration of 25 mg/ml caused pain and elicited a 2+ reaction in control subjects.

Five laboratory workers who had prolonged exposure to the material by inhalation (Group II) were tested. Three of these subjects had past history of other clinical allergies. None of the 5 subjects experienced symptoms upon inhalation of the bovine cartilage powder. Of this group one gave a 2+ positive in-

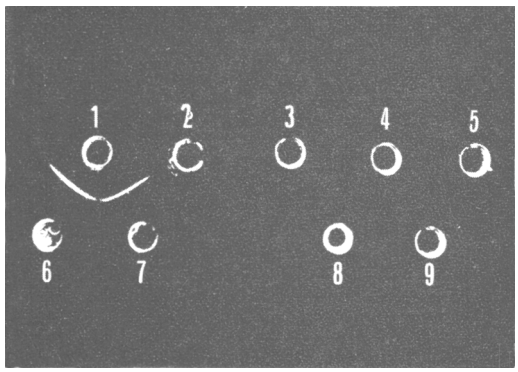

FIG. 2. Ouchterlony gel diffusion photograph. Wells #1,4 contain rabbit antisera against cartilage Lot A. Well #2—human cartilage, well #3—bovine serum, well #5—bovine pepsin, well #6—bovine cartilage lot B, well #7—bovine cartilage lot C, well #8—bovine hemoglobin, well #9—chrondroitin sulfate.

TABLE I

Patient group*	No. of subjects	Clinical allergy	Intradermal testing: Cartilage extract A	Bovine milk	Bovine epithelium	Bovine muscle	Bovine serum	Ragweed
I	5	0†	0					
II	5	3	1‡	0	0	0	1	2
III	9	1	0	0	1	0	0	1
IV	17	3	2‡	1	1	0	0	0

* See text.
† Number positive.
‡ Negative when tested with other cartilage lots.

tracutaneous test to the cartilage extract (Lot A). All of this group gave negative tests to bovine serum and aqueous extracts of bovine milk, epithelium, and muscle. The subject (JFP) with the positive test to cartilage extract from Lot A was then tested with extracts from 4 other lots and all were negative. Negative tests also resulted when this subject was tested with pepsin and other antigens: chondroitin sufate, N acetyl glucosamine and galactosamine.

Nine subjects received the cartilage on wounds which were excised after 7 to 14 days (Group III). Of this group, only one had a history of clinical allergy. None showed a positive reaction to the bovine cartilage extract, and testing with the other bovine antigens was completely negative except for a 1+ reaction to bovine epithelium in one patient. This patient gave no clinical history of allergic illness but also gave a 1+ reaction when tested with ragweed antigen.

Seventeen subjects received the cartilage during an operative procedure and the cartilage was not later removed (Group IV). The subjects were tested 2-18 months after application of the cartilage. Three of these subjects gave a positive past history of allergic illness. Testing with the bovine cartilage extract (lot A) yielded 1+ positive reactions in 2 subjects. Neither of these subjects gave positive reactions when tested with the other bovine antigens nor had they presented any allergic manifestations after exposure to the bovine cartilage. Both showed negative reactions when tested with 2 other cartilage lots. One patient with a history of episodic diarrhea showed a 3+ reaction to bovine milk extract but gave a negative reaction to bovine cartilage. One other subject showed a weakly positive test to bovine epithelium extract but was negative to the cartilage. All other subjects showed negative tests to all of the antigens of bovine origin (Table I). Ten subjects from this group were skin tested with 2 other lots of bovine cartilage extract and showed completely negative reactions. None of the subjects tested experienced a delayed reaction to the cartilage extract.

Ouchterlony gel diffusion was performed using the sera of 12 subjects from Group IV. The sera from the 2 subjects giving positive intradermal tests to cartilage Lot A were included as was the serum from the subject in Group II with a positive skin test. None of these sera yielded a positive precipitin reaction against bovine cartilage extract.

Discussion. Bovine cartilage material investigated here has been previously demonstrated to possess activity in accelerating wound healing.

In the use of any foreign material of animal origin for pharmacological purposes consideration must always be given to possible allergic reactions, especially with repeated administrations of the material.

Recently, processed bovine bone and cartilage have had extensive use in orthopedic and reconstructive surgery(16). The major antigenicity of this material was shown to reside through its bovine serum and red cell content. This antigenicity was removed by the the processing, thus markedly decreasing the problem of graft rejection; however, it was demonstrated that some specific bone antigenicity remained after the processing (17). No significant allergic reactions to this material have been reported.

The results of our experimentation showed

that rabbits did not form antibodies to the cartilage when exposed by implantation. This evidence would indicate that the material is of a low order of antigenicity. The rabbit immunization with the bovine cartilage emulsified with complete Freund's adjuvant did, however, demonstrate the production of precipitating antibodies, hence the material does possess some potential antigenicity under these artificial conditions. There was no cross reaction with other bovine antigens nor with human cartilage extract. This is in agreement with the work of DiFerrante and Pauling(18). In their study a hemagglutination technique was used and indicated that antibodies prepared against bovine proteinpolysaccharide of cartilage did not cross react with similar material prepared from human or porcine sources. A second antibody directed against chondroitin sulfate was also present and reacted with chondroitin sulfate prepared from other species. Their data seemed to indicate that the determinants responsible for the antigenicity of the proteinpolysaccharide resided in either the protein or keratin sulfate moieties.

Of 26 subjects in whom the material was used therapeutically, none experienced any symptoms which could be attributed to any allergic reaction to the material. However, 2 did produce weak skin sensitizing antibody activity to the material. This activity did not cross react with other bovine antigens tested.

Of 5 laboratory workers with a relatively large inhalation exposure to the bovine cartilage over a period of years, only one developed significant skin sensitizing antibody activity. This subject gave a history of other clinical allergies which were not exacerbated by the exposure to bovine cartilage powder, and no cross reactivity with other bovine products was found. It should be noted that the 3 subjects demonstrating weakly positive skin tests all did so to cartilage prepared from Lot A. This lot was one of the earlier preparations. Other cartilage lots are now processed so as to obtain purer preparations. Retesting the above subjects with three purer cartilage extracts yielded completely negative skin tests. It is not yet clear what was the contaminating material in the earlier lots.

The inability to demonstrate precipitating antibodies in patients formerly treated with the cartilage extract would seem to indicate that the preparation has weak antigenic activity in man, if it is antigenic at all. This would agree with the clinical experience obtained thus far with the use of bovine cartilage for reconstructive surgery.‖

It is of great importance that rabbit antibodies to bovine cartilage did not cross react with human cartilage or other bovine products. This is in agreement with the work of DiFerrante and Pauling(18). The lack of cross antigenicity should rule out the possibility of creating an autoimmune reaction to the patients own cartilage or sensitizing him to other bovine products.

With the repeated use of bovine cartilage material in the same individual, consideration must be given to the possibility that previous sensitization might have occurred. Previous work has shown that the two principle components of cartilage with antigenic potential, proteinpolysaccharide (chondromucoprotein) and chondroitin sulfate, are not active in accelerating wound healing(19). Thus one can hope that in the future a pure active material without any antigenic potential will be isolated from the cartilage extract.

Summary. A bovine cartilage preparation which has demonstrated therapeutic efficacy in accelerating wound healing in experimental animals and humans has been investigated for its potential antigenicity of the immediate type. Rabbits injected with an emulsion of this material with complete Freund's adjuvant did show the presence of precipitating antibodies while rabbits with implanted subcutaneous pellets showed no such activity. The precipitating antibodies produced did not cross react with human cartilage nor with other bovine tissues. Four groups of human subjects were tested intracutaneously with an aqueous extract of bovine cartilage. One laboratory worker with a history of clinical allergy and voluminous inhalation exposure of the material over a period of 8 years de-

‖ Squibb Boplant.

veloped skin sensitizing antibody activity. Mild activity was demonstrated by 2 subjects who had received the cartilage therapeutically by topical application. These 3 subjects had no symptoms that could be attributed to sensitivity reactions to the cartilage preparation and demonstrated negative skin reactions when tested with purer cartilage material prepared more recently. There was no cross reactivity with other bovine material tested. No activity was found in 24 subjects who had received the cartilage topically and in 4 laboratory workers who had long term inhalation exposure despite the fact that some of these subjects presented histories of previous clinical allergies. The sera from 12 of the subjects were examined for precipitating antibody to bovine cartilage and none was demonstrable. It is concluded that while the cartilage preparation does have some capacity for inducing antibody formation of the immediate type, on the basis of the above experiments this capacity appears relatively weak. Further purification of the active principle to eliminate all antigenic potential should be possible.

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