Acta Dermatovener (Stockholm) 52: 141–150, 1972

STUDIES ON THE MECHANISM OF ACTION OF ASIATICOSIDE (MADECASSOL®) ON EXPERIMENTAL GRANULATION TISSUE AND CULTURED FIBROBLASTS AND ITS CLINICAL APPLICATION IN SYSTEMIC SCLERODERMA

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Abstract. In an attempt to assess Madecassol® (containing asiaticoside, the active principal of *Centella asiatica,* a South African umbelliferous pennywort) for its effect on connective tissue, tests were made on the acid mucopolysaccharide content, L-glutamine fructose-6-phosphate transamidase activity and the collagen content of experimental carrageenin granulomas. Experiments were also conducted on morphological changes of cultured fibroblasts and ^{3}H-proline uptake of the cells. The results indicated that Madecassol inhibits the biosynthesis of acid mucopolysaccharides and collagens. The clinical use of Madecassol in systemic scleroderma brought symptomatic relief to 11 out of 13 patients, and improvement of laboratory test findings.

A South African umbelliferous pennywort, *Centella asiatica,* is widely known as a remedy for wounds by inhabitants of Madagascar and India. Madecassol, a preparation containing asiaticoside as the active principal of this herb, is currently used for the treatment of keloids and other skin diseases in France, South America, Egypt, and other countries (8).

The authors have recently used Madecassol for the treatment of patients with systemic scleroderma. The present paper describes the clinical results and the concomitantly conducted *in vitro* experiments to investigate its mechanism of action on fibroblasts.

I. EFFECT OF MADECASSOL ON FIBROBLAST METABOLISM

(a) *Acid Mucopolysaccharide Content, Mucopolysaccharide Synthetase Activity and Collagen Content of Carrageenin-granuloma*

Materials and Methods

Experimental carrageenin granuloma of guinea pigs was produced by Jackson's method (17). 5 ml of a 10% carrageenin solution in physiological saline was injected subcutaneously. Seven days later the granulation tissue formed at the injected site was removed for analysis. Madecassol, at a dose of 0.5 mg per 100 g body weight, was administered subcutaneously on the day preceding, and on the 1st, 3rd, and 5th days of carrageenin administration. Animals serving as controls received no drug or received prednisolone (Takeda Chemical Industries Ltd., Osaka, Japan), at a dose of 0.25 mg per 100 g body weight in the same manner as with the Madecassol injection.

The total acid mucopolysaccharide content of the carrageenin granuloma was determined according to the method of Kawamoto et al. (18). The granulation tissue was treated with pronase, dialysed, and precipitated with alcohol. CTAB (cetyltrimethylammonium bromide) was added to the precipitate, and the turbidity of the resulting acid mucopolysaccharide-CTAB complex was determined.

The activity of L-glutamine fructose-6-phosphate transamidase, one of the enzymes synthesizing acid mucopolysaccharide and being contained in the granuloma, was determined by the method of Ghosh et al. (11). The tissue homogenate prepared from the granuloma was centrifuged at 12 000 *g* for 30 min. The supernatant fluid was used as the enzyme solution. When estimating the specific activity of this enzyme, glucosamine and protein were determined by the methods of Rondle & Morgan (30) and Weichselbaum (39), respectively.

The collagen content of the granuloma was determined by the methods of Koevoet (21) and of Bergman & Loxley (2).

Results

The results are summarized in Table I. The acid mucopolysaccharide content in the granuloma (in μg per g tissue) in both Madecassol- and prednisolone-treated animals was significantly lower than in non-treated animals at the level of $p < 0.01$.

Whereas the non-treated animals exhibited some L-glutamine fructose-6-phosphate trans-

Table I. *Connective tissue constituents content in carrageenin granuloma of guinea pig*

	Acid mucopoly saccharides (μg/g tissue)	Specific activity of L-glutamine F-6-P transamidase (10^{-1} μmoles glucosamine produced per hour per mg protein)	Collagen mg/g tissue
Control group	951 ± 77^a $(6)^b$	66 ± 6^a $(5)^b$	19.0 ± 2.3^a $(7)^b$
Madecassoltreated group	601 ± 59 (5)	Trace	14.4 ± 1.9 (5)
Prednisolonetreated group	562 ± 66 (5)	Trace	14.0 ± 1.0 (5)

[a] Mean ± standard error.
[b] The number of animals studied.

amidase activity, both Madecassol- and prednisolone-treated animals showed no detectable enzymatic activity.

The collagen content in Madecassol- and prednisolone-treated animals was found significantly lower than in non-treated animals ($p < 0.01$).

(b) *Effects on the Growth of Cultured Fibroblasts and on Their Collagen-synthesizing Capacity*

Materials and Methods

Fibroblasts of human embryo skin were cultured at 37°C in Leighton tubes containing 20% bovine-serum Eagle MEM medium, and treated with trypsin. The cells were cultured in this medium alone or in the medium plus 10 μg/ml Madecassol or hydrocortisone. Cultured fibroblasts were taken at 1, 3, 8 and 24 hours of incubation, fixed with methanol and subsequently stained with Giemsa for microscopic examination.

In another experiment, cell cultures, to which ^{3}H-proline at a concentration of 5 μCi/ml had been added, were incubated for 1, 3, 8 and 24 hours, fixed with methanol, and autoradiographed by the dipping method using Kodak NTB-2. Amounts of the drugs added to the cell cultures were the same as in the preceding experiment.

Results

Media containing no drug (Fig. 1) exhibited satisfactory fibroblast and fibre proliferation after 24 hours' incubation. Media containing Madecassol (Fig. 2) or hydrocortisone (Fig. 3) inhibited proliferation of the cells markedly and produced partial degeneration.

In autoradiographic experiments on ^{3}H-proline uptake, the media without drugs showed blackening of numerous silver grains in the 3-hour cytoplasm (Fig. 4), in contrast to Madecassol- or hydrocortisone-containing media (Figs. 5 and 6, respectively), where cells were atrophied, and only a limited number of silver grains were seen in the cytoplasm.

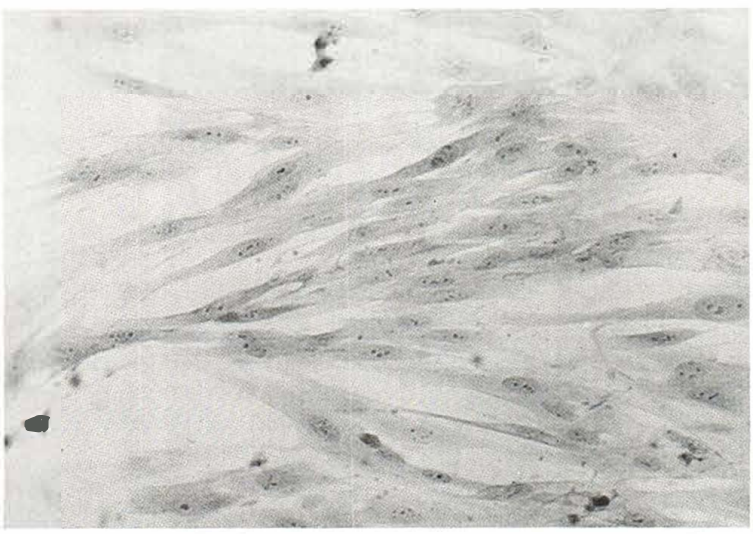

Fig. 1. Fibroblasts cultured without drug for 24 hours.

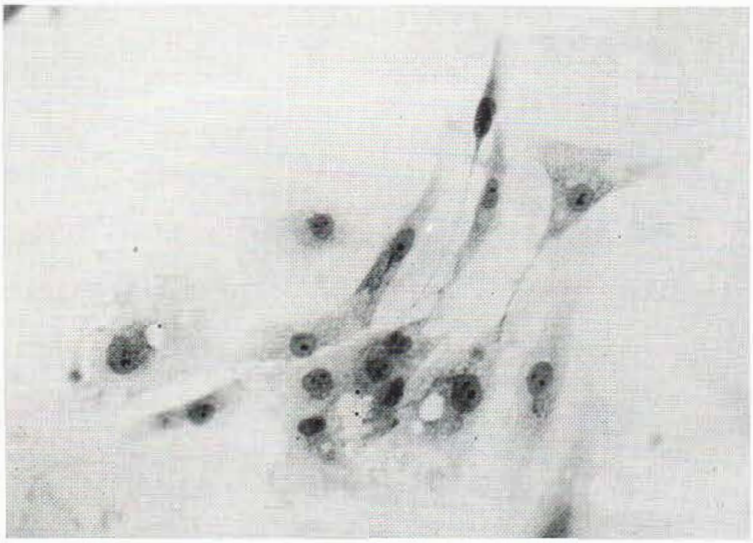

Fig. 2. Fibroblasts cultured with Madecassol for 24 hours.

II. CLINICAL RESULTS OF MADECASSOL IN THE TREATMENT OF SYSTEMIC SCLERODERMA

The subjects were 13 female in- and out-patients with systemic scleroderma treated at the Department of Dermatology, Kobe University Hospital. Most had been patients for 1 to 3 years and were in the sclerotic or atrophic stage.

Madecassol was administered either intramuscularly, 20 mg once or twice a week, or orally, 60 mg a day in 3 divided doses.

The concomitant use of other drugs was minimized.

Criteria of drug effect

In view of the extreme difficulty of assessing the effectiveness of drugs in the treatment of scleroderma, the authors employed the following arbitrary criteria in the evaluation of the present test drug: *excellent* when symptoms such as articular pain, motor disturbance, Raynaud's phenomenon, tautening and hardening of the skin, were alleviated markedly; *good* when symptoms were partly improved; *failure* when no improvement or even worsening of symptoms were observed.

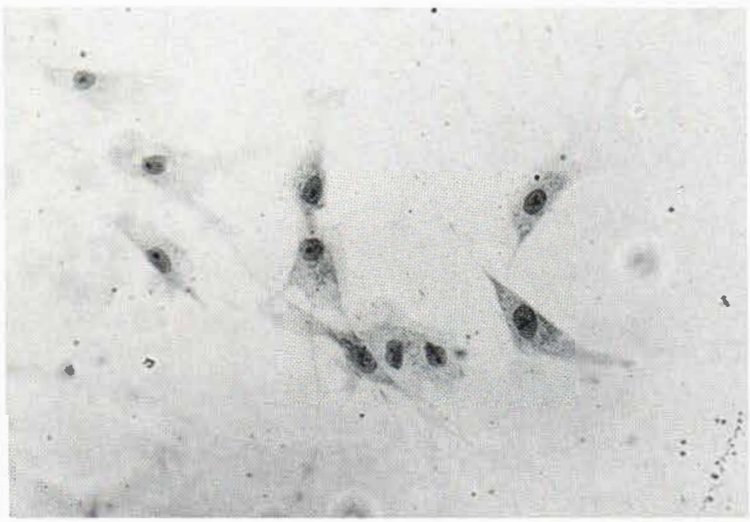

Fig. 3. Fibroblasts cultured with hydrocortisone for 24 hours.

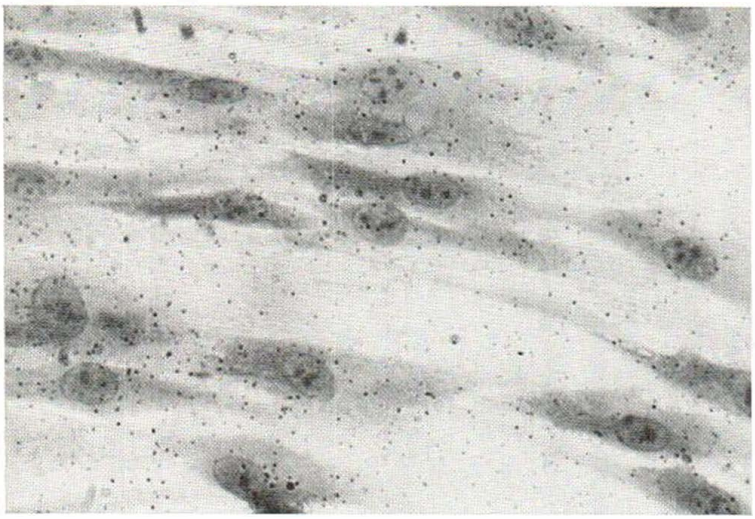

Fig. 4. ^{3}H-proline autoradiography of fibroblasts cultured without drug for 3 hours.

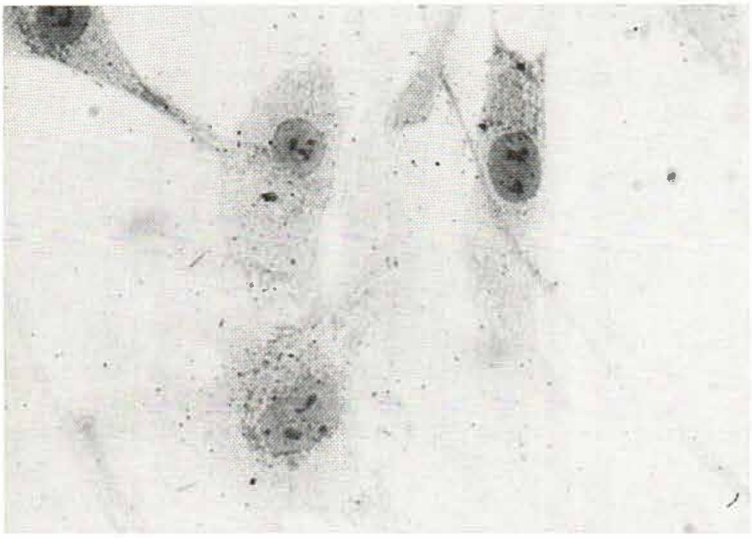

Fig. 5. ^{3}H-proline autoradiography of fibroblasts cultured with Madecassol for 3 hours.

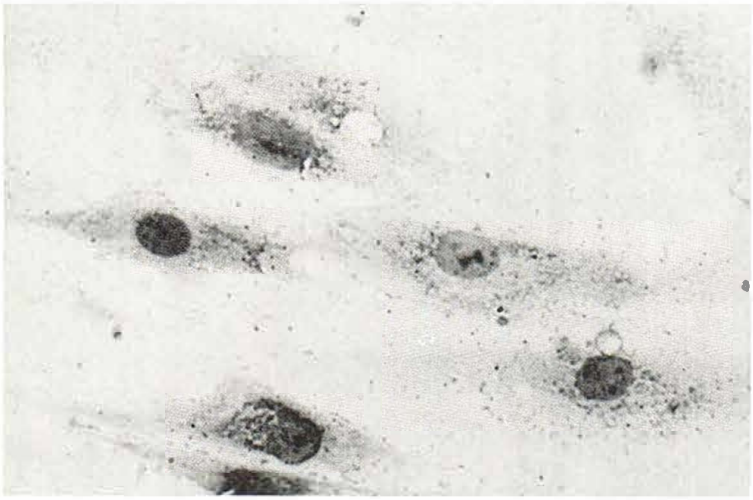

Fig. 6. ^{3}H-proline autoradiography of fibroblasts cultured with hydrocortisone for 3 hours.

Table II. *Clinical results of Madecassol in patients with systemic scleroderma*

R.P. = Symptoms of Raynaud's phenomenon

Case no.	Age	Sex	Time elapsed from onset to first examination	Clinical symptoms at first examination	Dosage & duration	Clinical course	Remarks	Result
1	32	♀	12 y.	So-called CRST syndrome; pain in joints of extremities & motor disturbance	I.M. once per 1–2 w. for 1.5 y.	Motor disturbance relieved markedly; hardening of skin of whole body remitted but ulcerations formed on fingers; gain in weight		Good
2	25	♀	8 y.	Hardening of skin of whole body, particularly of face and extremities; tips of fingers & toes deformed with positive Raynaud's phenomenon (R.P.)	I. M. once a week for 8 mo. P.O. 6 tab. per day for 6 mo.	With 8 inj. the skin became less hardened and general condition improved: lassitude persisted and R.P. hung in the balance		Good
3	43	♀	4–5 y.	Cyanosis and sclerosis of finger tips: the skin of face taut and stretched	I.M. once a week for 5 mo. P.O. (6 tab/d) for 1 mo.	In 2 mo. of therapy hardening of skin of finger tips markedly reduced; tautness of facial skin disappeared; the symptoms hung in the balance thereafter	Slight fever	Good
4	41	♀	4 mo.	R.P. in periphery of extremities, hardening of skin & pain	I.M. once a week for 8 mo.	After 5 mo. of therapy (18 inj. doses) hardening of skin reduced markedly; R.P. almost disappeared	Slight fever	Excellent
5	16	♀	8 mo.	Hardening of skin of left lower extrem., multiple small patches of depigmentation, R.P. (+)	I.M. once per 5 d. for 5 mo.	In 2 mo. (15 inj.) the skin became somewhat less hardened; R.P. subsided		Good
6	47	♀	10 mo.	Swelling & slight sclerosis of upper extrem., notably forearms	I.M. once a week for 4 mo.	In 3 mo. (9 inj.) hardening of skin relieved		Good
7	54	♀	1 y.	Acrocyanosis, generalized hardening of skin, some difficulty in mouth-opening	I.M. once a week for 2 mo. I.M. twice weekly for 1 mo.	Unchanged	Depot male hormone inj. following oophorectomy for cancer	None
8	36	♀	2 y.	R.P. & hardening of skin of trunk and forearms conspicuous	I.M. once a week for 6 mo.	Became able to skip rope; cutaneous change reduced except in right forearm		Good
9	35	♀	3 y.	R.P. of finger tips, pronounced systemic hardening of skin	I. M. once a week for 3 mo.	Symptoms almost unchanged. (21 inj. doses in total)		None
10	16	♀	3 y.	Acrocyanosis, hardening of skin, swelling & tautness of face	I.M. biweekly for 5 mo.	In 2 mo. (7 inj.) facial swelling & hardening of finger tips subsided		Excellent
11	45	♀	2 y.	R.P. of tips of fingers & toes, systemic hardening of skin	I.M. once a week for 6 mo.	In 3 mo. (18 inj.) hardening of skin reduced, but general malaise prominent	Slight fever	Good

Table II (*continued*)

Case no.	Age	Sex	Time elapsed from onset to first examination	Clinical symptoms at first examination	Dosage & duration	Clinical course	Remarks	Result
12	38	♀	2 y.	Sclerosis & atrophy of skin of whole body; patchy depigmentation of face, neck & shoulders; R.P. (+)	I.M. once a week for 10 mo.	Hardening & atrophy of skin stopped progressing; general condition quite good; R.P. somewhat relieved		Good
13	19	♀	1.5 mo.	Swelling & tenderness on dorsal aspect of all fingers; weakness of entire upper extrem.	I.M. once per 10 d. for 1.5 mo.	In 1.5 mo. (4 inj.) both swelling and weakness disappeared		Excellent

Results

Table II summarizes the mode of administration and dosage of the drug, the signs, symptoms and course of the disease. Duration of the treatment ranged from 1.5 months to 1.5 years. All the patients except cases 2 and 3 received the drug intramuscularly.

Excellent or good response was found in 11 patients, accounting for the effectiveness rate of 84.6%. The patients required 2 or 3 months (i.e. 10 to 20 injections) before experiencing reduced skin tautness and definite relief from circulatory disorders, except for some patients whose hardened skin and motor disturbance abated after only a few injections. Laboratory findings and pulmonary function were satisfactory.

The course of a 25-year-old patient (case 2) is illustrated in Table III. Therapy with this drug resulted in not only much improved symptoms but also definitely improved findings in ASLO, CRP and other serological tests, and pulmonary function tests.

Table III. *Results of laboratory examinations before and after therapy with Madecassol (case no. 2)*

	Before therapy		After therapy
(1) Blood sedimentation rate	Apr., 1969	July, 1969	Dec., 1969
60 min	17	66	54
120 min	71	105	85
(2) Blood count	Jan., 1969	Sept., 1969	Dec., 1969
RBC ($\times 10^4$)	408	407	426
Hemoglobin (g/dl)	11.6	10.1	10.0
WBC	5 700	8 400	5 000
Platelets ($\times 10^4$)	20.8	16.9	15.4
Differential count (%)			
Neutrophils rod	28	10	17
With 2 lobulated nuclei	42	66	35
With 3 lobulated nuclei	15	11	23
Monocytes	2	2	1
Lymphocytes	13	11	24
(3) Immunohematology	Apr., 1969	Aug., 1969	Dec., 1969
RA	—	++	++
ASLO	50 T.U.	166 T.U.	50 T.U.
CRP	++++	+++	++
(4) Pulmonary function test		July, 1969	Dec., 1969
Vital capacity (ml)		830	1 170
Vital capacity (%)		29	40.3
Maximal breathing capacity (l/min)		26.4	30.1
Timed vital capacity		750	940

Table IV. *Results of pulmonary function test before and after administration of Madecassol (case no. 4)*

	Before therapy March, 1970	After therapy Sept., 1970
Vital capacity (ml)	2 110	2 020
Vital capacity (%)	75.4	72.2
Residual volume (%)	67.6	41.3
Total lung capacity (%)	178	94
Maximal breathing capacity (l/min)	39	49.6
Timed vital capacity	79.6	71.1

Table IV shows the results of pulmonary function tests before and after administration of Madecassol in a 41-year-old patient (case 4). It revealed a considerable improvement of % residual volume, % total lung capacity as well as of maximal breathing capacity, though the vital capacity and timed vital capacity both remained almost unchanged.

Fig. 7 illustrates how the abnormally low serum level of collagen-like protein (CLP) of this patient returned to normal. Serum levels of collagen-like protein of a 16-year-old patient (case 5) are presented in Fig. 8, which shows that low collagen-like protein levels returned to normal during administration of this drug.

Two patients were regarded as failures. One of these, case 9, stayed away after a 3 month follow-up period, in which the symptoms remained unchanged. Another patient, case 7, suffered from supervened ovarian cancer, and metastases were found in other organs and lymph nodes on laparotomy.

In spite of prolonged administration of Madecassol, no toxic effect was evidenced on laboratory examination or on scrutiny of clinical signs.

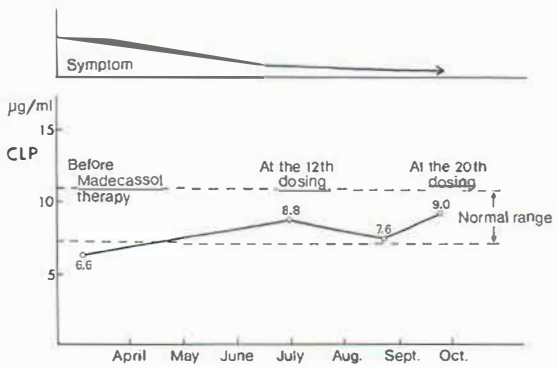

Fig. 7. Changes in serum CLP in a patient with systemic scleroderma receiving Madecassol (case 4).

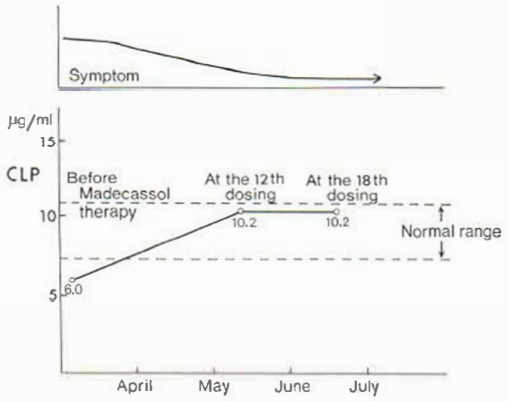

Fig. 8. Changes in serum CLP in a patient with systemic scleroderma receiving Madecassol (case 5).

DISCUSSION

Asiaticoside was first isolated by Bontems in 1941 (5), and its chemical structure (Fig. 9) was determined by Polonsky et al. (28). The primary pharmacologic action of this drug was elucidated by Boiteau & Ratsimamanga (3). Since then, numerous experiments have been conducted on the promotion and regulation of the healing

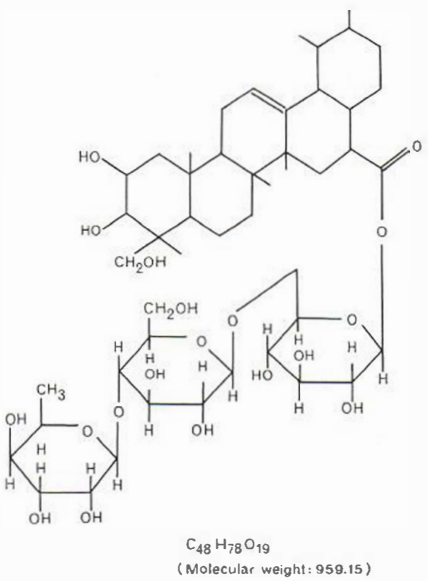

Fig. 9. Chemical structure of asiaticoside. (Main ingredient of centella asiatica extract.)

process of wounds as well as the activation of cellular elements of mesenchymal and ectodermal tissues (23, 24).

In the present study, the authors studied the action of the drug on connective tissue, and recognized the reduced content of mucopolysaccharides and collagen in carrageenin granulomas, as well as an inhibited activity of mucopolysaccharide synthetase therein. Biochemical, morphological, and autoradiographic studies of cultured fibroblasts were in keeping with the inhibition of fibre formation by this agent. These findings clearly demonstrate that Madecassol suppresses biosynthesis of the ground substance and collagen fibres of the connective tissue essentially in the same way as do adrenocortical hormones (prednisolone and hydrocortisone). As has been pointed out by many authors (1, 9, 32), adrenocortical hormones inhibit the growth of fibroblasts. It is likely that this action contributes to the stabilization of the lysosomal membrane, and leads to interference with lysosomal enzyme release. Our findings suggest that Madecassol is similar to corticoids, at least in its behaviour toward the synthesis of the connective tissue. Though asiaticoside, the active principal of Madecassol, resembles corticosteroids somewhat in chemical structure, it seems unlikely that it exerts corticoidal action *in vivo*.

In the present clinical trial, the drug was used with the intention of treating systemic scleroderma, and proved to be effective. The following are our comments on its mechanism of action.

Many explanations have been given of the etiology and pathogenesis of systemic scleroderma, among which the auto-immunization theory has recently gained wide acceptance. Burnet (6) and Kierland (19) described how a rise in γ-globulin is one of the aids to the diagnosis of auto-immune diseases. Sasaki & Clausen (34) reported on elevated values of IgG, IgA and IgM; Hall (12) on antinuclear antibodies; and Steffen (38) on antigenicity exerted by the connective tissue itself. Meanwhile, Shinkai (35) recognized that sera from patients with scleroderma have a cytolytic effect on cultured fibroblasts. Elucidation of the relation between his findings and the pathogenesis of this disease, however, awaits further studies. Studies so far have failed to demonstrate the presence of specific antibodies to the connective tissue of the patients, but pointed out only vaguely that an immunity mechanism is implicated in the cause of this disease.

Although the etiology and pathogenesis of scleroderma still remain unknown, considerable attention has been paid to the changes of the connective tissue. Sclerotic changes of the vasculature, followed by the deposition of glycoproteins, have been found to occur in the early stages of the disease. It has been widely accepted that, despite the thinning of the collagen bundles, the fibres themselves remain unchanged when examined electron-microscopically (31).

Recently, glycoprotein and mucopolysaccharide constituting the ground substance of the connective tissue have attracted much attention. As to the content of mucopolysaccharide and one of its constituents, hexosamine, in sclerodermic skin, various results have been reported. Korting et al. (22) found that the hexosamine content falls within the normal range; Noda (27) found the total mucopolysaccharide content to be normal or increased; and Sasaki (33) found it definitely increased. Such a discrepancy may be attributable to different stages of the disease studied.

As to the composition of acid mucopolysaccharides, both Noda (27) and Sasaki (33) pointed out an increased proportion of chondroitin sulfate B, and Ishikawa (16) demonstrated the presence of an unusual, unidentified sulfated mucopolysaccharide in the initial stages. Bollet et al. (4) and Rodnan (29) reported elevated levels of uronic acid, while Fleischmajer (10) reported an increase in hexosamine levels in blood serum of scleroderma patients. Denko & Stoughton (7) demonstrated an increased production of sulfated mucopolysaccharides from the observed increase of ^{35}S-uptake; Holzmann et al (15) reported an increased urinary excretion of acid mucopolysaccharides.

Sjoerdsma et al. (36) and Smith et al. (37) stated that the urinary level of hydroxyproline falls within the normal range in subjects affected with scleroderma, while Korting et al. (22) reported a reduced content of total collagen and a remarkably increased content of neutral salt-soluble collagen in sclerodermic skin. Holzmann et al. (13) described how the administration of gestagen accelerates the degradation of neutral salt-soluble collagen and thereby increases its urinary excretion.

In 1964, hydroxyproline in blood was classified

by LeRoy et al. (25) into two major classes, the free type and the peptide type. The latter is called collagen-like protein (CLP) and considered as representing collagen or its denatured product. Therefore, the level of CLP serves as an important index for collagen metabolism in the body.

According to Holzmann et al. (14) and Kishihara et al. (20), the serum level of CLP in patients with scleroderma is definitely lower than that of normal persons. Holzmann et al. (14) stated that remission of symptoms caused by gestagen therapy was accompanied by a normalized CLP value. The presumed action exerted by gestagen is acceleration of soluble collagen degradation, which is prompted by the enhanced activities of proteolytic enzymes such as cathepsine and leucine aminopeptidase (26). It is assumed that Madecassol gives rise to a similar mode of action, but this assumption must await further studies. Nevertheless, if one takes into account the fact that Madecassol interferes with the biosynthesis of collagen and mucopolysaccharide, the use of this drug for systemic scleroderma becomes justifiable.

ACKNOWLEDGEMENT

Madecassol® (a product of Laboratoires Laroche Navarron, Levallois-Paris, France) was generously supplied by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan.

REFERFENCES

1. Asboe-Hansen, G.: Hormone control of connective tissue. Federation Proc *25:* 1136, 1966.
2. Bergman, I. & Loxley, R.: Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. Analyt Chem *35:* 1961, 1963.
3. Boiteau, P. & Ratsimamanga, A. R.: L'asiaticoside, extrait de « Centella Asiatica » et ses emplois thérapeutiques dans la cicatrisation des plaies expérimentales et rebelles. Thérapie *11* (1): 125, 1956.
4. Bollet, A. J., Scraydarian, M. W. & Simpson, W.: Acid mucopolysaccharides in normal serum. J Clin Invest *36:* 1328, 1957.
5. Bontems, J.: Bull. Sci Pharmacol *49:* 186, 1941.
6. Burnet, F. M.: Autoimmune disease-experimental and clinical (Jephcott Lecture). Proc Roy Soc Med *55:* 619, 1962.
7. Denko, C. W. & Stoughton, R. B.: Fixation of ^{35}S in the skin of patients with progressive systemic sclerosis. Arthr Rheum *1:* 77, 1958.
8. El-Hefnawi, H.: Treatment of keloid with asiaticoside. Dermatologica *125:* 387, 1962.
9. Fisher, E. R. & Paar, J.: Carrageenin granuloma in the guinea pig and rat. I. Effect of hydrocortisone, estradiol and mast cell depletion on its histological and histochemical features. Arch Path *70:* 556, 1960.
10. Fleischmajer, R.: Serum proteins and glycoproteins in scleroderma. Arch Derm (Chicago) *89:* 749, 1964.
11. Ghosh, S., Blumenthal, H. J., Davidson, E. & Roseman, S.: Glucosamine metabolism. V. Enzymatic synthesis of glucosamine 6-phosphate. J Biol Chem *235:* 1265, 1960.
12. Hall, A. P., Bardawil, W. A., Bayles, T. B., Mednis, A. D. & Galins, N.: The relations between the antinuclear, rheumatoid and L.E. cell factors in the systemic rheumatic diseases. New Engl J Med *263:* 769, 1960.
13. Holzmann, H., Korting, G. W. & Morsches, B.: Zur Therapie der Sklerodermie mit Gestagenen. Hautarzt *16:* 456, 1965.
14. Holzmann, H., Korting, G. W., Morsches, B. & Schlandecker, A.: Zum Verhalten des „Collagen-like Protein" im Serum von Sklerodermie-Kranken vor und nach Therapie mit Gestagen. Arch Klin Exp Derm *230:* 69, 1967.
15. Holzmann, H., Korting, G. W. & Morsches, B.: Zur Beeinflussung der Mucopolysaccharide in Serum und Urin von Sklerodermiekranken durch Gestagen-Behandlung. Arch Klin Exp Derm *231:* 156, 1968.
16. Ishikawa, E.: Studies on cutaneous acid mucopolysaccharides in patients with systemic scleroderma. Reported at the 2nd General Meeting of the Connective Tissue Researches, Tokyo, 1970.
17. Jackson, D. S.: Connective tissue growth stimulated by carrageenin. Biochem J *65:* 227, 1957.
18. Kawamoto, S., Sato, Y. & Hasegawa, E.: The determination of acid mucopolysaccharide of paranasal sinus membrane by cetyltrimethylammonium bromide reagent. J Clin Oto-Laryn *19:* 612, 1966.
19. Kierland, R. R.: Associations of systemic lupus erythematodes, scleroderma and dermatomyositis. Jap J Dermat, Ser. A., *76:* 504, 1966.
20. Kishihara, Y., Sasaki, S. & Sano, S.: Studies on serum collagen-like protein in some dermatoses. Reported at the 21st Annual Meeting of Mid Jap Derm, Kyoto, 1970.
21. Koevoet, A. C.: The determination of hydroxyproline in urine. Clin Chim Acta *12:* 230, 1965.
22. Korting, G. W., Holzmann, H. & Kühn, K.: Biochemische Bindegewebsanalysen bei progressiver Sklerodermie. Klin Wschr *42:* 247, 1964.
23. Lawrence, J. C.: The morphological and pharmacological effects on asiaticoside upon skin in vitro and in vivo. Europ J Pharmacol *1:* 414, 1967.
24. — The effect of asiaticoside on guinea pig skin. J Invest Derm *49:* 95, 1967.
25. LeRoy, E. C., Kaplan, A., Udenfriend, S. & Sjoerdsma, A.: A hydroxyproline-containing, collagen-like protein in plasma and a procedure for its assay. J Biol Chem *239:* 3350, 1964.
26. Morsches, B., Holzmann, H., Korting, G. W. & Braun, M.: Zur Einfluss von bindegewebswirksam

Substanzen auf den Leucin-aminopeptidase-Gehalt im Ratten Serum. Arzneimittel-Forsch *16:* 1081, 1966.
27. Noda, M.: Studies on fractional determination of the acid mucopolysaccharides in the cutaneous tissues. Jap J Dermat, Ser. A., *75:* 609, 1965.
28. Polonsky, J. Sach, E. & Lederer, E.: Sur la constitution chimique de la partie glucidique de l'asiaticoside. Bull Soc Chim France, *6:* 880, 1959.
29. Rodnan, G. P.: A review of recent observations and current theories on the etiology and pathogenesis of progressive systemic sclerosis (diffuse scleroderma). J Chron Dis *16:* 929, 1963.
30. Rondle, C. J. M. & Morgan, W. T.: The determination of glucosamine and galactosamine. Biochem J *61:* 586, 1955.
31. Rupec, M. & Braun-Falco, O.: Elektronenmikroskopische Untersuchungen über das Verhalten der Kollagenfibrillen der Haut bei Sklerodermie. Arch Klin Exp Derm *218:* 543, 1964.
32. Sasaki, S.: Effects of hormones on the cutaneous connective tissue. Skin Res (Osaka) *12:* 125, 1970.
33. — Observations on acid mucopolysaccharide synthesis of the skin. Reported at the 34th Annual Meeting of East Jap Derm Assoc (Lecture), Tokyo, 1970.
34. Sasaki, S. & Clausen, J.: An immunological study on mastocytosis. Acta Dermatovener (Stockholm), *49:* 382, 1969.
35. Shinkai, H.: The effects of anti-collagen serum on the cultured fibroblast. Connective Tissue, *2:* 11, 1970.
36. Sjoerdsma, A., Udenfriend, S., Keiser, H. & LeRoy, E. C.: Hydroxyproline and collagen metabolism. Ann Int Med *63:* 672, 1965.
37. Smith, Q. T., Rukavina, J. G. & Haaland, E. M.: Urinary hydroxyproline in various diseases. Acta Dermatovener (Stockholm), *45:* 44, 1965.
38. Steffen, C.: Antigenicity and autoantigenicity of collagen. Ann NY Acad Sci *124:* 570, 1965.
39. Weichselbaum, T. E.: An accurate and repid method for the determination of proteins in small amounts of blood serum. Amer J Clin Path Tech, *Suppl. 16:* 46, 1945.

Received July 21, 1971

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