Phytomedicine Vol. 2 (1), pp. 17–22, 1995

In vivo wound healing activity of Dragon's Blood (*Croton* spp.), a traditional South American drug, and its constituents

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Summary

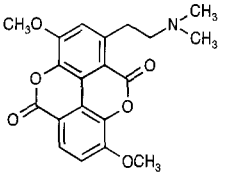
The wound healing activity of dragon'sblood (*Croton* spp.), in Spanish ‚sangre de drago' or ‚sangre de grado', a traditional South American drug, and some of its constituents, including the alkaloid taspine (1), the dihydrobenzufuran lignan 3',4-O-dimethylcedrusin (2) and proanthocyanidins, was evaluated *in vivo* on rats, and compared with the wound healing actitivy of synthetic proanthocyanidins. The beneficial effect of dragon's blood on wound healing was confirmed. Dragon's blood stimulated contraction of the wound, formation of a crust, formation of new collagen, and regeneration of the epithelial layer. 3',4-O-Dimethylcedrusin also improved wound healing *in vivo* by stimulating the formation of fibroblasts and collagen, but crude dragon's blood was more effective. This was due to the proanthocyanidins, present in dragon's blood, which stimulate contraction of the wound and precipitate with proteins forming a dark crust covering the wound, but which delay wound repair by a decreased formation of new fibroblasts.

Key words: *Croton* spp., dragon's blood, proanthocyanidins, wound healing.

Introduction

Dragon's blood, in Spanish ‚sangre de drago' or ‚sangre de grado', is a traditional South American drug used for several purposes, including wound healing. This blood-red, viscous latex is extracted from various *Croton* spp. (Euphorbiaceae), including *C. lechleri* L., *C. draconoides* (Muell.) Arg. (or *C. palanostigma* Kl.), and *C. erythrochilus* (Muell.) Arg., by slashing the bark (Ubillas et al. 1994). The alkaloid taspine (1) was reported to be the active cicatrizant principle (Vaisberg et al. 1989). The bioassay-guided fractionation of dragon's blood, using an *in vitro* test system for stimulation of human endothelial cells as a model for wound healing (Vanden Berghe et al. 1993), resulted in the isolation of a dihydrobenzofuran lignan, 3',4-O-dimethylcedrusin (2) as the active principle (Pieters et al. 1993). *In vivo* guiding tests on laboratory animals for the isolation of the wound healing principles from dragon's blood were excluded for practical and ethical reasons. In the present study the *in vivo* wound healing activity on rats of dragon's blood and some of its constituents, including taspine, 3',4-O-dimethylcedrusin and polyphenolic compounds (proanthocyanidins), which can account for up to 90% of the dried weight of dragon's blood (Cai et al. 1991), is reported, and compared with the wound healing activity of synthetic proanthocyanidins.

(1)



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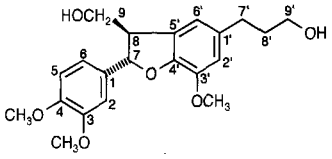

Experimental

Materials. Dragon's blood, obtained from Peruvian *Croton* spp., was purchased in liquid form from Quimica Uni-

versal, Lima, Peru. The isolation of taspine (1) and 3',4-O-dimethylcedrusin (2) was reported previously (Pieters et al. 1993). The polyphenolic fraction, containing the proanthocyanidins, was prepared by extracting 100 ml of dragon's blood, acidified with citric acid (2%), with Et_2O (4 x 100 ml) to remove 3'-4-O-dimethylcedrusin, and next with a (1:1) mixture of EtOAc/n-BuOH (4 x 100 ml). The EtOAc/n-BuOH extract was washed with HCl 1 N (4 x 200 ml), and evaporated to dryness (polyphenolic fraction, 6.8 g). In order to produce dimeric or oligomeric proanthocyanidins by organic synthesis, a condensation reaction of taxifolin and catechin was carried out under nitrogen. The reaction mechanism is shown in fig. 1. 2 g of (±)-taxifolin (racemic taxifolin) (Sigma) were dissolved in 50 ml of EtOH. 50 ml of a 4% solution of $NaBH_4$ (Janssen Chimica) were added over a period of 40 min, in order to effect the complete reduction of taxifolin. Next 5 g of (+)-catechin (Sigma), dissolved in 35 ml of EtOH, were added, the pH of this solution was brought to about 4 with glacial HAc, and next to pH about 1 with concentrated HCl. After stirring for about 5 hours at room temperature under nitrogen, 100 ml of H_2O were added, and the reaction mixture was extracted with EtOAc (3 x 250 ml). The EtOAc extract, containing the polyphenolic compounds, was concentrated under reduced pressure, washed with H_2O, and evaporated to dryness. This residue was analysed by tlc on silica gel using EtOAc/H_2O,HCOOH/HAc (75/20/3/2) as the mobile phase. After spraying with 5% H_2SO_4 in EtOH and 1% vanillin in MeOH, and heating the plates to 120 °C, phenolic compounds developed red-brown spots (Kolodziej, 1986; De Bruyne, 1995).

In vivo experiments. The *in vivo* experiments to evaluate the wound healing activity were carried out on female Wistar rats (250–300 g), supplied with food and water *ad libitum*, and maintained in separate cages. The rats were anesthetized with Hypnorm (100 µl/100 g body weight), shaved and disinfected with 75% EtOH. Circular excised wounds were made by cutting off the epidermis of the rat's back, and then 2 ml of boiling distilled water were applied to the wounds. Treatment started 1 hour after the injury was made, and consisted in applying about 0.5 ml of an ointment or solution containing the product or fraction to be tested, twice daily for 18 days, and next once a day. The result of each treatment was compared with untreated rats, and rats treated with a placebo ointment. Each treatment was tested on a pair of two rats. Wound healing was examined daily during the experiment and pictures were taken on regular times. Especially the area and depth of the lesions and the evolution of the healing process in terms of tissue contraction and crust formation were accurately followed up.

In a first *in vivo* experiment on rats, carried out as described above, 3',4-O-dimethylcedrusin was applied as a 0.1% solution in polyethylene glycol 400 (PEG 400) 10%, and taspine hydrochloride as a 0.01% solution in distilled water. Wounds were about 1 cm in diameter (four wounds on each rat). Only one product was tested on each rat (on two wounds), the other wounds were used for a placebo treatment and for a control experiment (untreated wound). After 10 days of treatment all rats were sacrificed, the wound area of each rat was carefully removed, and maintained in 4% formaldehyde at 4 °C. Slices were prepared for microscopic evaluation.

In a second *in vivo* experiment, carried out as described above, wounds were about 3 cm in diameter (one wound on each rat). 3',4-O-dimethylcedrusin was applied as a 0.05% ointment. A polyethylene glycol (PEG) ointment, which consisted of PEG 4000 (29.5%), PEG 400 (53%), cetyl alcohol (4.2%) and water (13.3%) was used. Taspine was tested at two different concentrations, including 0.05% (the same concentration as the 3',4-O-dimethylcedrusin ointment), and 0.005% (because of the *in vitro* cytotoxicity, as observed before (Pieters et al. 1993), using the same PEG ointment. After one month of treatment all rats were sacrificed, and slices of the wound areas were prepared for microscopic evaluation as described above.

In a third experiment, the wounds were about 2 cm in diameter (two wounds on each rat). 3',4-O-dimethylcedrusin was applied as a 0.0014% ointment, and taspine as a 0.09% ointment, which corresponded to their actual concentration in dragon's blood (Pieters et al. 1993), using the same PEG ointment as in the first experiment. The polyphenolic fraction from dragon's blood was applied as a 7% solution in distilled water (since 6.8 g of this fraction were isolated from 100 ml of dragon's blood, corresponding to a concentration of about 7%). The result of this treatment was compared with rats treated with a 7% solution of synthetic polyphenolic compounds, prepared as described above. After only 15 days of treatment all rats were sacrificed and slices of the wound areas were prepared for microscopic evaluation as described above.

In all *in vivo* experiments, each treatment was compared with untreated rats, rats treated with a placebo, and rats treated with crude dragon's blood.

Results and discussion

The wound healing activity of dragon's blood and some of its constituents was evaluated in three *in vivo* experiments on rats. Macroscopic and microscopic observations made during these *in vivo* experiments are summarized in tables 1, 2 and 3, respectively. From a macroscopic point of view, the wound healing process was evaluated in terms of (1) contraction of the wound after the first day of treatment, (2) formation of a crust, and (3) wound repair, being the % of the wound (as a volume, not as a surface) filled with newly formed tissue. (see Tables 1–3) From a microscopic point of view, tissue repair at the end of the experiment (at the moment the slices were prepared) was evaluat-

ed in terms of (1) newly formed tissue, (2) epithelial growth (formation of a new epithelial layer), and (3) formation of new hair follicles. (see Tables 1–3) In order to evaluate the formation of new tissue, this process was divided into three consecutive stages. Stage 1 is characterised by the formation of new blood vessels at the edge of the wound, migration of white blood cells, formation of fibroblasts and formation of a crust. Stage 2 is characterised by the formation of blood vessels, concentration of white blood cells under the crust, and the presence of a lot of fibroblasts and only few collagen; a crust is formed. In stage 3 only few blood vessels are left, collagen and epithelial tissue are formed. Toxicity of the product or the vehiculum applied is observed by a decrease in the formation of new blood vessels, and/or the formation of fibroblasts, and/or the formation of collagen from fibroblasts, and/or a delay in the formation of new epithelial tissue. The stage of the wound healing process at the moment the microscopic slices were prepared depends on the initial diameter of the wound, time of treatment (length of the experiment) and the treatment itself. Since new hair follicles are only formed at the end of the wound healing process, this criterion could only be used in the second experiment, which was run over one month, and not in the first and the third experiment, where the healing process evolved over a shorter period of time. Essentially, the results are evaluated semiquantitatively. A quantitative approach, allowing statistical evaluation, would require the simultaneous treatment of a high number of laboratory animals; this was excluded for ethical reasons.

From tables 1–3 it can be concluded that crude dragon's blood clearly has a beneficial effect on wound healing (experiments I.3, II.5 and III.4) as compared to appropriate control experiments (I.5, II.6 and III.7). Water can be considered as the vehiculum of dragon's blood; the effects of preparations containing herbal drug are clearly different from vehiculum effects. After only one day of treatment contraction of the wound was observed and the complete wound area was covered with a dark crust. It appeared that dragon's blood stimulated contraction of the wound, formation of a crust and formation of new collagen, and that the epithelial layer regenerated quickly and completely. Since after only 1 day of treatment the complete wound was covered with a dark crust, this phenomenon may have a physical rather than a biochemical origin. In order to understand this, it must be realized that up to 90% of the dried weight of dragon's blood consists of polyphenolic compounds (proanthocyanidins), derived mainly from gallocatechin and epigallocatechin. In addition to (+)-catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin, and dimeric procyanidins B-1 (epicatechin-(4β-8)-catechin) and B-4 (catechin-(4α-8)-epicatechin), several other dimeric

Table 1. Results of the first *in vivo* experiment.

No.	TREATMENT	MACROSCOPIC OBSERVATIONS			MICROSCOPIC OBSERVATIONS	
		contraction[a]	formation of a crust[b]	wound repair[c]	new tissue[d]	epithelial growth[e]
I.1	3',4-O-dimethylcedrusin 0.1% in PEG 400 10%	0	after 5 days	80%	2	++
I.2	Taspine HCl 0.01% in H_2O	0	after 4 days	77%	2	+
I.3	Dragon's blood	++ (1 day)	after 1 day	95%	3	++
I.4	PEG 400 10%	0	after 5 days	80%	2	++
I.5	H_2O	0	after 4 days	70%	2	++
I.6	Untreated	+ (1 day)	after 3 days	65%	2	++

Table 2. Results of the second *in vivo* experiment.

No.	TREATMENT	MACROSCOPIC OBSERVATIONS			MICROSCOPIC OBSERVATIONS		
		contraction[a]	formation of a crust[b]	wound repair[c]	new tissue[d]	epithelial growth[e]	new hair follicles[f]
II.1	3',4-O-dimethylcedrusin 0.05% in PEG ointment	0	after 5 days	85%	2–3	+++	++
II.2	Taspine 0.05% in PEG ointment	0	after 5 days	80%	2	+	0
II.3	Taspine HCl 0.005% in PEG ointment	0	after 5 days	85%	2	+	++
II.4	PEG ointment	0	after 5 days	80%	2	+	0
II.5	Dragon's blood	+++ (1 day)	after 1 day	95%	3	++++	++++
II.6	H_2O	0	after 4 days	85%	2	+	+
II.7	Untreated	+ (1 day)	after 3 days	85%	2	+	0

and trimeric proanthocyanidins were isolated and identified as catechin-(4α-8)-epigallocatechin, gallocatechin-(4α-8)-epicatechin, gallocatechin-(4α-6)-epigallocatechin, catechin-(4α-8)-gallocatechin-(4aα-8)-gallocatechin, and gallocatechin-(4α-8)-gallocatechin-(4α-8)-epigallocatechin. Higher oligomers were also obtained (Cai et al., 1991). When applied to an open wound, these polyphenols may precipitate proteins, and form an artificial crust covering the wound.

In order to test this hypothesis treatments with the polyphenolic fraction from dragon's blood and a synthetic polyphenolic fraction were evaluated separately. The polyphenolic fraction from dragon's blood was applied as a solution, at the same concentration as in crude dragon's blood. However, it should be noted that this polyphenolic fraction contains relatively more lower oligomers than crude dragon's blood, since they are more easily extracted by EtOAc/n-BuOH than the higher oligomers. The polyphenolic fraction from dragon's blood (experiment III.5) stimulated contraction of the wound and formation of a crust, but it delayed wound repair by a decreased formation of new fibroblasts. It had no influence on the growth of new blood vessels. Newly formed tissue was mature and epithelial tissue was formed, but the wound was not completely filled.

In the oligomeric proanthocyanidins isolated from dragon's blood, an exceptionally high content of gallocatechin and epigallocatechin is oberserved (Cai et al., 1991). Proanthocyanidins derived mainly from gallocatechin and epigallocatechin (prodelphinidins), such as in dragon's blood, are rather rare in nature. The majoritiy of oligomeric and polymeric proanthocyanidins found so far have a higher content of catechin and epicatechin (procyanidins). Treatment with the polyphenolic fraction from dragon's blood was compared with rats treated with a solution of synthetic procyanidins (mainly dimers), having the same concentration. These procyanidins were prepared by a condensation reaction of (±)-taxifolin (racemic taxifolin) and (+)-catechin. The reaction product mainly consisted of dimeric procyanidins (Fig. 1), a minor quantity of trimeric procyanidins, a large amount of residual catechin and traces of residual taxifoline. Starting from 2 g of taxifolin, the theoretical maximum yield is about 4 g of dimeric or 3 g of trimeric procyanidins, since only the lower terminal unit of synthetic oligomeric procyanidins is derived from catechin. 4 different dimeric procyanidins may be obtained by condensation of racemic (±)-taxifolin and (+)-catechin, including (+)-catechin-(4α-8)-(+)-catechin (or procyanidin B-3, see Fig. 1), (+)-catechin-(4α-6)-(+)catechin (or procyanidin B-6), (−)-catechin-(4β-8)-(+)-catechin, and (−)-cate-

Table 3. Results of the third *in vivo* experiment.

No.	TREATMENT	MACROSCOPIC OBSERVATIONS			MICROSCOPIC OBSERVATIONS	
		contraction[a]	formation of a crust[b]	wound repair[c]	new tissue[d]	epithelial growth[e]
III.1	3',4-O-dimethylcedrusin 0.014% in PEG ointment	0	after 4 days	85%	2	+
III.2	Taspine 0.09% in PEG ointment	0	after 4 days	75%	2	+
III.3	PEG ointment	0	after 4 days	70%	2	0
III.4	Dragon's blood	+++ (1 day)	after 1 day	90%	3	++
III.5	Polyphenolic fraction from Dragon's blood in H_2O	++ (1 day)	after 1 day	50%	T	++
III.6	Synthetic polyphenols in H_2O	+ (1 day)	after 1 day	40%	T	+
III.7	H_2O	+ (1 day)	after 4 days	60%	2	+

Legend for tables 1–3

a contraction: 0 = no contraction
+ to +++ = increasing level of contraction

b formation of a crust: a first crust is formed on the wound after the given number of days

c wound repair: % of the wound volume filled with new tissue, observed after biopsy

d new tissue: 1 to 3 = stage of formation of new tissue (see text)
T = toxic

e epithelial growth: 0 = no epithelial growth
+ to ++++ = increasing level of epithelial growth

f new hair follicles: 0 = no hair follicles
\+ = few introversions
++ = obvious introversions
+++ = more obvious introversions
++++ = introversions with beginning differentiation

chin-(4β-8)-(+)-catechin. It is known that in this type of condensation reactions 4–8 linked procyanidins are obtained in a much larger yield (about 10 to 1) than their 4–6 linked isomers, because of the lower steric hindrance in case of a 4–8 linkage. Therefore, procyanidin B-3 and its diastereomer (–)-catechin-(4β-8)-(+)-catechin can be considered to be the principal reaction products. Approach by the nucleophilic (+)-catechin is presumably favored from the less hindered side of the 4-carbonium ion, thus predominantly leading to a 3,4-*trans* stereochemistry, as in naturally occurring proanthocyanidins. The dimeric compounds are more nucleophilic than the monomers. Therefore, in order to obtain higher yields of trimeric procyanidins, less (+)-catechin, relative to taxifoline, should be used (Kolodziej, 1986; De Bruyne, 1995).

When applied to a wound, a solution of synthetic polyphenols (with the same concentration as in dragon's blood) experiment III.6) formed a film covering the wound and stimulating contraction, but it had a negative influence on wound repair. The formation of new fibroblasts was inhibited, and only few new tissue was formed. New epithelial cells were present.

Macroscopic evaluation of the wound healing process revealed that those wounds treated with crude dragon's blood, the polyphenolic fraction from dragon's blood, and the synthetic proanthocyanidins, were almost immediately (*i.e.*, on day 1) covered with a crust. When treated with dragon's blood itself, this crust was thick and dark, with the polyphenolic fraction from dragon's blood thick and rather brown, but when treated with the synthetic proanthocyanidins it was only a thin, brown film, which gradually grew thicker. This indicated that the synthetic polyphenols, belonging to the class of the procyanidins, basically have the same effect as the prodelphinidins from dragon's blood, and that the polyphenolic fraction from dragon's blood was indeed responsible for the formation of this thick, dark crust. The synthetic proanthocyanidins basically showed the same effect, although less pronounced. This may be due to the fact that the higher oligomers present in dragon's blood are more effective than dimeric compounds.

3',4-O-Dimethylcedrusin had a beneficial effect on wound healing when applied in a PEG ointment (experiments II.1 and III.1), as compared to appropriate control experiments (II.4 and III.3): It stimulated the formation of fibroblasts and collagen, and the wound was filled with new, mature tissue. This effect was similar to that observed for dragon's blood, but less pronounced. 3',4-O-Dimethylcedrusin in PEG 400 10% or in a PEG ointment delayed contraction of the wound and formation of a crust, but a comparison of, e.g., experiment I.1 with I.4, I.5 an I.6 showed that this was due to the vehiculum. Wound repair, however, was not influenced. In general, as compared to untreated wounds, contraction is delayed by covering the wound with an aqueous solution or an ointment, except for dragon's blood and the polyphenolic solutions, which stimulate contraction. Covering a wound with a PEG ointment results in the formation of a thick, weak crust.

Taspine had no influence on the wound healing process (experiments I.2, II.2, II.3 and III.2), even at high, cytotoxic concentrations (Pieters et al. 1993). During *in vivo* experiments taspine may be biologically complexated and inactivated.

Neither the formation of new blood vessels, nor the migration of white blood cells is influenced by any of the treatments. The proliferation of fibroblasts is stimulated by dragon's blood and 3',4-O-dimethylcedrusin, but inhibited by the polyphenolic fractions. This inhibition results in a decreased formation of new tissue. The turn-over from fibroblasts to collagen is stimulated by dragon's blood and 3',4-O-dimethylcedrusin. This maturation of new tissue results in a faster healing process. The formation of new epithelial tissue is stimulated by dragon's blood and, to a lesser extent, by the polyphenolic fractions. After treatment with dragon's blood, as compared to untreated animals, a tendency to the formation of new hair follicles is observed.

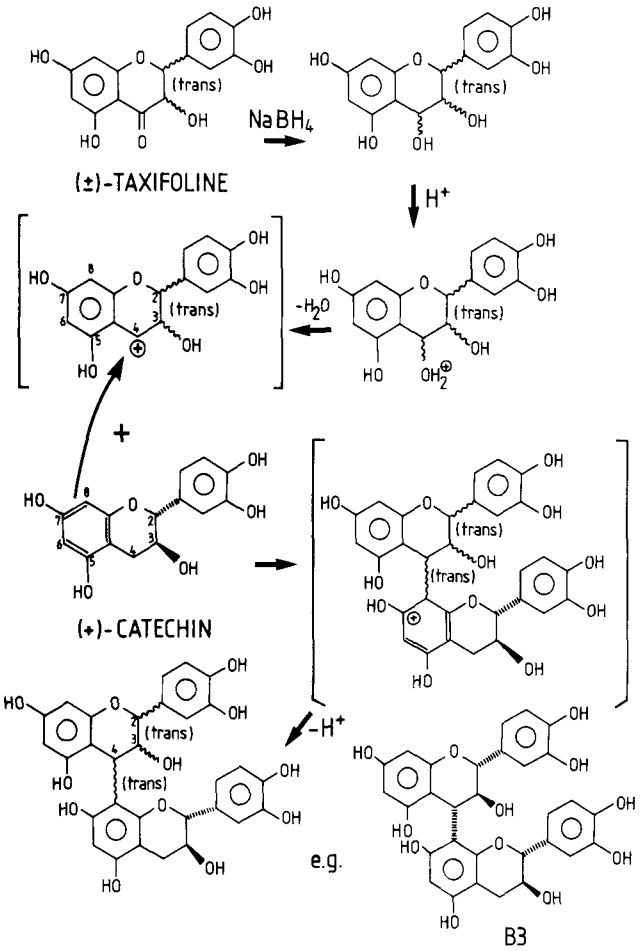

Fig. 1: Condensation of racemic (±)-taxifolin and (+)-catechin

At the end of the experiments, when the laboratory animals were sacrificed to prepare slices of the wound areas for microscopic evaluation, it appeared that treatment with dragon's blood resulted in wound healing without scars, in contrast to untreated animals (of course further healing after a prolonged period could not be excluded).

The experiments on wound healing as described above provide a good model for the evaluation of the wound healing activity of test compounds. The wound healing process is influenced by the diameter of the wounds, the vehiculum of the test compounds, and duration of the treatment. Mechanical influences such as scratching cannot be excluded and may interfere with the evaluation of the healing process. Microscopic evaluation of slices prepared from the wound areas is possible only once during the healing process, and results in the end of the experiment. Therefore, a continuous follow-up of the healing process is only possible by macroscopic evaluation. In none of the laboratory animals inflammation was observed during the experiment. None of the animals showed an aggressive behaviour, which leads us to the conclusion that the experiments never were irritant or painful. It should be stressed that the number of *in vivo* experiments was strictly limited to a minimum, since the bioassay guided fractionation of dragon's blood was carried out using *in vitro* model for wound healing (Vanden Berghe et al., 1993; Pieters et al. 1993). However, when wound healing is concerned, an *in vivo* confirmation of biological activity is essential.

In conclusion, *in vivo* experiments on rats to evaluate the wound healing activity of dragon's blood, 3',4-O-dimethylcedrusin and taspine, confirmed the beneficial effect on wound healing of crude dragon's blood, which is used for this purpose in traditional South American medicine. Dragon's blood stimulated contraction of the wound, formation of a crust, formation of new collagen, and regeneration of the epithelial layer. 3',4-O-Dimethylcedrusin, which was identified as the biologically active principle by *in vitro* bioassay-guided isolation, also improved wound healing *in vivo* by stimulating the formation of fibroblasts and collagen. The wound healing effect of crude dragon's blood was better than that observed for ointments containing 3',4-O-dimethylcedrusin. This was due to the proanthocyanidins, present in dragon's blood, which stimulate contraction of the wound and precipitate with proteins forming a dark crust covering the wound, although the lower oligomers may delay wound repair by a decreased formation of new fibroblasts. These results could be helpful as indicators of potential use allowing to devise further trials with a quantitative approach and statistical analysis.

Acknowledgements

This work was supported by grant no. 92/94-09 of the Flemish Government (Belgium). T. De Bruyne received a fellowship from the University of Antwerp (UIA) (1994–1996).

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