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Prolonged inhibition of luteinizing hormone and testosterone levels in male rats with the luteinizing hormone-releasing hormone antagonist SB-75

(inhibitory analogs of luteinizing hormone-releasing hormone/gonadal inhibition/microgranules)

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ABSTRACT **Inhibitory effects of the potent antagonist of luteinizing hormone-releasing hormone *N*-Ac-[3-(2-naphthyl)-D-alanine[1],4-chloro-D-phenylalanine[2],3-(3-pyridyl)-D-alanine[3], D-citrulline[6],D-alanine[10]]luteinizing hormone-releasing hormone (SB-75) free of edematogenic effects were investigated in male rats. In a study to determine the effect on luteinizing hormone levels in castrated male rats, SB-75 was injected s.c. in doses of 0.625, 1.25, 2.5, 5.0, and 10 μg. Blood samples were taken at different intervals for 48 hr. All doses of SB-75 significantly decreased luteinizing hormone levels for >6 hr ($P < 0.01$); this inhibition lasted for >24 hr ($P < 0.01$) with a dose of 5.0 μg and >48 hr with 10 μg ($P < 0.05$). Serum testosterone levels were also measured in intact male rats injected with SB-75 in doses of 25, 50, and 100 μg. All doses produced a dramatic fall in testosterone to castration levels 6 hr after injection ($P < 0.01$); this inhibition of serum testosterone was maintained for >72 hr, but only the 100-μg dose could keep testosterone in the castration range for >24 hr ($P < 0.01$). In another study using a specific RIA, we obtained the pharmacokinetic release pattern of SB-75 from two sustained delivery formulations of SB-75 pamoate microgranules and examined their effect on serum testosterone. After a single i.m. injection of 20 mg of one batch of microgranules, a large peak corresponding to SB-75 at 45.8 ng/ml was observed, corresponding to the "burst" effect. Levels of the analog decreased to 19.6 ng/ml on day 2, gradually reached a concentration of 4.7 ng/ml on day 7, and kept declining thereafter. Testosterone levels were reduced on day 1 ($P < 0.01$) and were maintained at low values for >7 days ($P < 0.05$). In rats injected with 10 mg of SB-75 pamoate microgranules of the second batch, SB-75 serum levels rose to 33 ng/ml 3 hr after administration and then fell gradually to ≈3.4 ng/ml on day 16, but a second small peak was seen on day 28. Subsequently, the analog levels decreased slowly to 2.9 ng/ml on day 42. At this time, testosterone serum levels were still significantly lower than in controls. These overall results demonstrate the efficacy of SB-75 in the suppression of the pituitary–gonadal axis. This modern luteinizing hormone-releasing hormone antagonist can possibly be used for treating sex hormone-sensitive cancers and other disorders.**

Since 1971, >3000 analogs of hypothalamic luteinizing hormone-releasing hormone (LH-RH) have been synthesized for possible medical application (1–4). Although repeated chronic administration of LH-RH agonists is required to inhibit luteinizing hormone (LH) and follicle-stimulating hormone release and reduce the levels of sex steroids, similar effects can be obtained with a single dose of LH-RH antagonists (1–4).

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LH-RH superagonists have been used clinically, both to promote and to inhibit fertility and for the treatment of some steroid-dependent tumors (1, 3, 4). However, progress in development and clinical application of LH-RH antagonists has been slow. High-dose requirements, due to low potency of earlier compounds and side effects related to histamine release (1–5) of analogs with D-arginine in position 6, delayed clinical use of LH-RH antagonists.

LH-RH antagonists, free of edematogenic and anaphylactoid reactions, containing neutral hydrophilic D-ureidoalkyl amino acids such as D-citrulline and D-homocitrulline at position 6, were recently synthesized in our laboratory and tested *in vitro* and *in vivo* (6, 7). Among these analogs, *N*-Ac-[3-(2-naphthyl)-D-alanine[1],4-chloro-D-phenylalanine[2], 3-(3-pyridyl)-D-alanine[3],D-citrulline[6],D-alanine[10]]LH-RH (SB-75) was shown to be one of the most powerful antagonists in inhibiting ovulation (7)—blocking ovulation completely in normal cycling rats at a 2-μg dose (7). Furthermore, when SB-75 was compared with other antagonists, it showed practically no release of histamine from peritoneal mast cells *in vitro* (7).

Since 1983, we have been developing sustained-delivery systems for peptides. These systems are based on microcapsules or microgranules of polymers of DL-lactide-coglycolide, which are biodegradable and compatible with living tissues (4, 8–11). We have demonstrated the efficacy of formulations of microcapsules of the LH-RH agonist [D-Trp[6]]LH-RH, somatostatin analog RC-160, and more recently, our LH-RH antagonist SB-75 (4, 8–11). More modern forms of sustained-delivery systems, which consist of microparticles (microgranules) containing SB-75 pamoate or [D-Trp[6]]LH-RH have been also prepared (12). While microcapsules are more uniform spherical particles with a diameter of 40–50 μm, microparticles are amorphous with a wide variety of sizes, ranging from 5 to 80 μm (12). These microgranules are obtained by cryogenic grinding of extruded polymer containing the homogeneously dispersed peptide and do not involve solvents like freon, which may be banned on environmental grounds in the future.

In the present paper we describe the inhibitory effects of our LH-RH antagonist SB-75 on gonadotrophin and sex steroid secretion in male rats. We also report a pharmacokinetics study of the release pattern of SB-75 from two other formulations of SB-75 pamoate microgranules.

MATERIAL AND METHODS

Animals. Male Sprague–Dawley rats (Charles River Breeding Laboratories) weighing 280–420 g were used in all experiments. Animals were allowed standard rat diet and tap

Abbreviations: LH, luteinizing hormone; LH-RH, LH-releasing hormone.

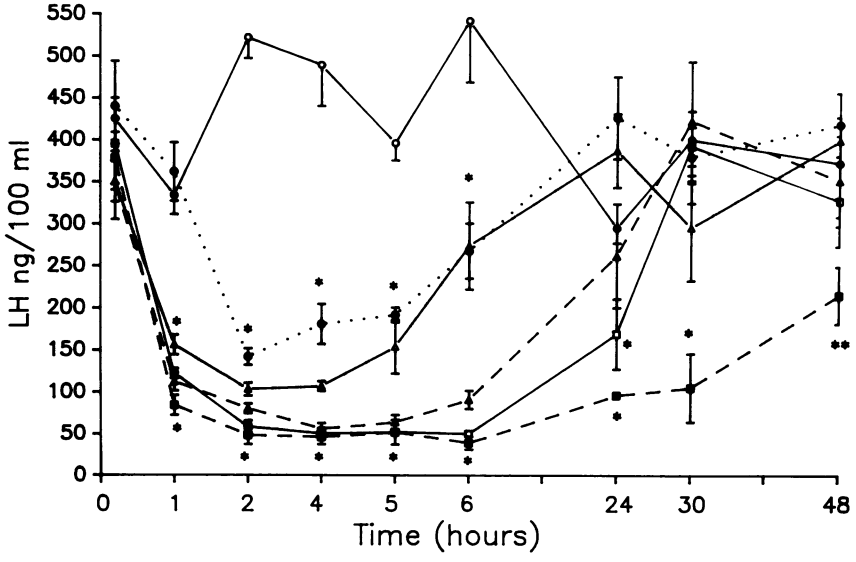

FIG. 1. LH serum levels in castrated male rats after s.c. injection of SB-75 in doses of 0.625 (●), 1.25 (△), 2.5 (▲), 5.0 (□), and 10 μg (■); control dose is (○). **, $P < 0.05$ and *, $P < 0.01$ vs. control group.

water ad libitum and were maintained under controlled conditions: 12 hr light/12 hr dark schedule at 24 ± 2°C.

Peptide and Microgranules. *N*-Ac-[3-(2-naphthyl)-D-alanine[1],4-chloro-D-phenylalanine[2],3-(3-pyridyl)-D-alanine[3],D-citrulline[6],D-alanine[10]]LH-RH (SB-75) was synthesized by solid-phase methods in our laboratory as well as by Asta-Degussa (Frankfurt/Main, F.R.G.) and carefully repurified by HPLC (7). SB-75 pamoate microgranules lots RGS 0001 and RGS 9002 were prepared by P. Orsolini (Cytotech, Martigny, Switzerland) by using a described method (12) and consisted of SB-75 pamoate [1.5% and 4.5% (wt/wt), respectively] distributed within a polymer matrix of poly(DL-lactide-coglycolide). For injection, the microgranules were suspended in 0.7 ml of injection vehicle consisting of 2% CM-cellulose and 1% Tween 80 in water (10, 11). The suspension was mixed thoroughly on a Vortex mixer and injected i.m. through an 18-gauge needle.

Experiment 1. Rats castrated 2 weeks earlier were anesthetized with urethane at 150 mg per 100 g of body weight (Sigma) and 60 min later SB-75 dissolved in saline was injected in doses of 0.625, 1.25, 2.5, 5.0, and 10.0 μg per rat; control animals were injected only with saline. Blood samples were drawn from the jugular vein before and 1, 2, 4, 5, 6, 24, 30, and 48 hr after peptide administration to determine LH levels.

Experiment 2. Intact rats anesthetized with Metofane (Pitman-Moore, Washington Crossing, NJ) were injected with SB-75 in doses of 25, 50, and 100 μg per rat, while control animals received only saline. Blood samples were collected from the jugular vein before dosing and 6, 24, 48, 72, and 96 hr after the peptide injection for measurement of testosterone levels.

Experiment 3. Microgranules of SB-75 pamoate (lot RGS 0001 and lot RGS 9002) in aliquots of 20 mg and 10 mg, respectively, were injected into young adult male rats. Control animals received only vehicle injection. Ten rats were used in each group. Blood samples of 250 μl were taken from the tail vein into capillary tubes during the first week and three times a week thereafter. The serum was separated by centrifugation, and the supernatant was diluted 1:1 with phosphate buffer containing 0.5% sodium azide. At the end of the experiment, animals injected with lot RGS 9002 were sacrificed, and testicles, ventral prostate, and seminal vesicles were removed and weighed.

Hormone Determination. Serum LH was determined by specific RIA with material supplied by the National Hormone and Pituitary Program (13). Testosterone levels were measured with a kit provided by Diagnostic Products (Los Angeles). SB-75 was determined by RIA by using a highly specific antibody developed in our laboratory (14). All samples for each hormone were analyzed together in the same assay. Intraassay variation was <10%, and interassay coefficient of variation was <15%. The results are expressed as mean ± SEM. Statistical significance was assessed by Duncan's new multiple-range test (15).

RESULTS

The effects of different doses of SB-75 on LH levels in castrated male rats are shown in Fig. 1. All doses markedly decreased LH levels 1 to 2 hr after injection ($P < 0.01$). With lower doses (0.625–2.5 μg), the inhibition of LH was maintained for >6 hr ($P < 0.01$) (Fig. 1), whereas with higher doses the fall in LH lasted for >24 hr with 5 μg ($P < 0.01$) and >48 hr for 10 μg ($P < 0.05$) (Fig. 1).

When testosterone levels were measured in intact rats 6 hr after SB-75 administration, we observed that the doses of 25–100 μg markedly decreased this hormone to castration levels ($P < 0.01$) (Fig. 2). Although 24 hr after administration all groups had lower testosterone levels than controls

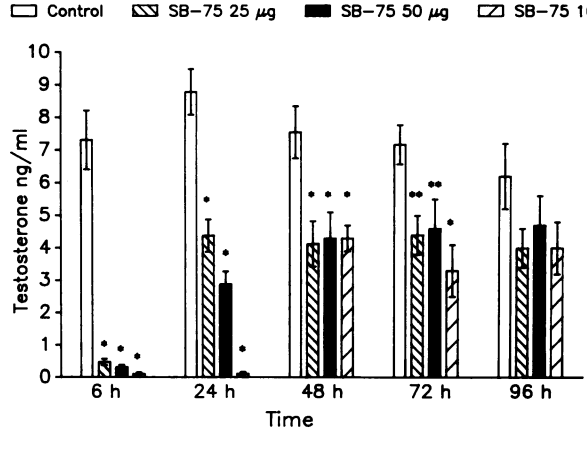

FIG. 2. Testosterone levels in male rats after s.c. injection of LH-RH antagonist SB-75 in doses of 25, 50, and 100 μg. **, $P < 0.05$ and *, $P < 0.01$ vs. control group.

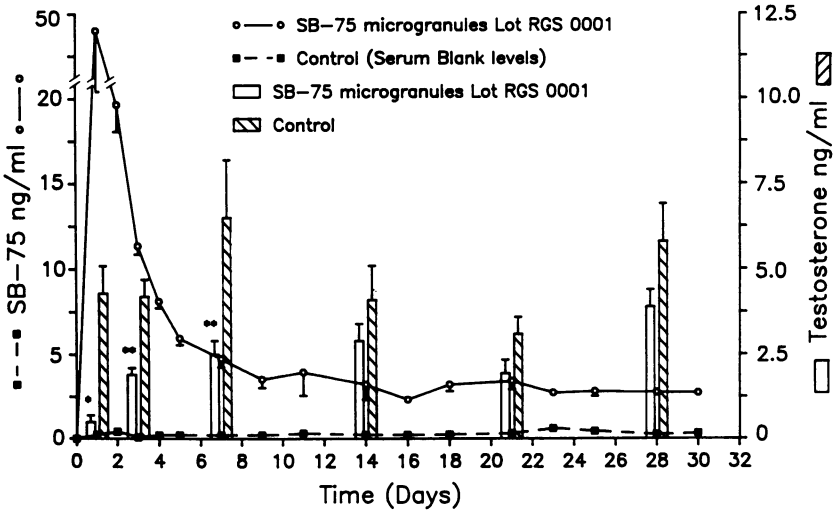

FIG. 3. The curve represents serum SB-75 levels in male rats injected with 20 mg of SB-75 microgranules lot RGS 0001. Serum blank levels of controls are indicated by the dashed line. The bars show serum testosterone levels and SB-75 microgranules in injected animals and in controls evaluated on days 1, 3, 7, 14, 21, and 28. **, $P < 0.05$ and *, $P < 0.01$ vs. controls.

($P < 0.01$), only the animals injected with 100 μg of SB-75 showed values in the castration range ($P < 0.01$) (Fig. 2). All the doses maintained significantly lower levels of this hormone for >72 hr ($P < 0.05$ or $P < 0.01$) (Fig. 2).

SB-75 levels in serum of rats injected with a single dose of 20 mg of SB-75 microgranules lot RGS 0001 are shown in Fig. 3. Twenty hours after injection, a large peak, corresponding to 45.8 ng/ml was detected, indicating a "burst" effect. The peptide levels decreased to 19.6 ng/ml on day 2 and gradually reached a concentration of 4.7 ng/ml on day 7. The SB-75 levels kept declining until day 16 to 2.3 ng/ml and then showed a very small increase to 3.2 and 3.4 ng/ml on day 18 and 21, respectively (Fig. 3). The levels of the antagonist kept declining thereafter on days 23–30. After administering these microgranules, testosterone levels decreased significantly on day 1 ($P < 0.01$) and were lower than controls for >7 days ($P < 0.05$). From days 14 to 28 hormone levels did not differ between control and treated animals.

In rats injected with 10 mg of SB-75 pamoate microgranules, lot RGS 9002, serum SB-75 levels rose to 33 ng/ml in 3 hr and then kept falling gradually to ≈3.4 ng/ml on day 16 (Fig. 4). This level was maintained until day 21, and then SB-75 concentrations gradually rose to 7.3 ng/ml on day 28. Subsequently, levels of the analog decreased slowly to 2.9 ng/ml on day 42 (Fig. 4), but this level was still significantly higher ($P < 0.01$) than the serum blank measured in the control group. After administering SB-75 microgranules from this batch, testosterone serum levels fell to castration levels during the first 24 hr ($P < 0.01$) and were maintained significantly lower than controls until day 42 ($P < 0.01$ and $P < 0.05$) (Fig. 4), except for day 28, when because of a transient decrease in testosterone levels in controls, hormone concentrations did not differ between treated and untreated rats (Fig. 4). The effects of SB-75 microgranules lot RGS 9002 on body, testicular, ventral prostate, and seminal vesicles weight are shown in Table 1. Forty-two days after injection, ventral prostate weight had decreased in treated animals ($P < 0.01$). At this time, no changes were found in the weight of other organs (Table 1).

DISCUSSION

Chronic administration of superactive agonists of LH-RH produces a marked inhibitory effect through downregulation of receptors and desensitization of pituitary gonadotrophs

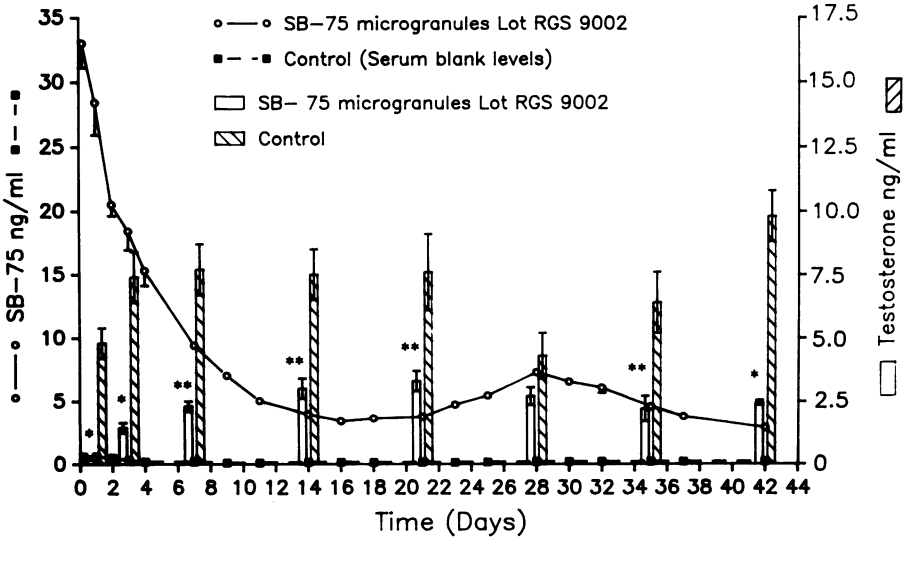

FIG. 4. The curve represents serum SB-75 levels in male rats injected with 10 mg of SB-75 microgranules lot RGS 9002. Serum blank levels of controls are indicated by dashed line. The bars show testosterone serum levels and SB-75 microgranules in injected animals and in controls evaluated on days 1, 3, 7, 14, 21, 28, 35, and 42. **, $P < 0.05$ and *, $P < 0.01$ vs. controls.

Table 1. Body, testicular, ventral prostate, and seminal vesicle weights and serum LH levels in male rats treated with SB-75 microgranules evaluated 42 days after injection

	Weight, g					
	Body					
	Initial	Final	Testicles	Ventral prostate	Seminal vesicles	Serum LH, ng/ml
Control	307 ± 4.4	459 ± 9.1	3.2 ± 0.06	0.42 ± 0.02	1.41 ± 0.03	0.83 ± 0.09
Treated	304 ± 4.0	465 ± 10	3.0 ± 0.06	0.31 ± 0.02*	1.33 ± 0.07	0.58 ± 0.05

SB-75 microgranules were from lot RGS 9002.
*$P < 0.01$ vs. control.

(1–4). This inhibition of the pituitary–gonadal axis is manifested by a decrease in the secretion of LH, follicle-stimulating hormone, and sex steroids (1–4). These effects impart to the LH-RH agonists great therapeutical value. They may be used for the control of human fertility (1, 3, 4) and for the treatment of gynecologic disorders (1, 3, 4) and hormone-sensitive tumors, such as prostate and breast cancer (1, 3, 4, 15). Although repeated administration of LH-RH agonists is necessary for the inhibition of gonadal functions, similar effects can be induced with a single injection of a potent LH-RH antagonist (1–7). During administration of LH-RH agonists, a transient release of LH and sex steroids that precedes the hormonal inhibition may result in a flare-up in disease. Because the LH-RH antagonists induce immediate suppression, they should prevent these phenomena, which can occur in some cancer patients (4).

Antagonists of LH-RH have been originally proposed for use in contraception (1, 2, 4, 5). However, when highly potent antagonists, containing arginine in position 6, were injected s.c. into rats at doses of 1.25–1.5 mg/kg, they caused transient edema of the face and extremities. On i.v. injection into rats, these analogs could induce cyanosis and respiratory depression leading to death (5–7, 16). We have synthesized additional LH-RH antagonists, free of edematogenic effects and other anaphylactoid reactions in rats and humans‡ (6, 7). Among these analogs, SB-75 emerges as one of the most powerful (7). Our present results prove that SB-75 is a highly potent antagonist of LH-RH, as evidenced by the inhibition of LH levels in castrated rats in doses as small as 0.625 μg and by marked suppression of testosterone to castration levels in intact rats. In both cases, duration of the inhibition was directly related to the dose administered. The doses of 25–100 μg of SB-75 led to significantly lower testosterone levels than those in control animals for >72 hr, although only the 100-μg dose maintained testosterone at castration levels for >24 hr, indicating a protracted effect by the antagonist. Similar sustained inhibitory actions on gonadotropin secretion were reported in ovariectomized monkeys and in postmenopausal women with higher doses of the earlier antagonists (17–19).

We have demonstrated that administration based on the use of sustained delivery systems such as microcapsules or osmotic minipumps is much more efficacious than multiple daily injections (10, 11, 20, 21). Daily administration of SB-75 was significantly less effective in inhibiting gonadal function in male mice than the treatment based on the use of minipumps for the delivery of this antagonist (21). In addition, microcapsules of SB-75 have been shown to powerfully inhibit the growth of experimental prostate cancers in rats (E. Korkut, L.B., K.G., and A.V.S., unpublished results). Furthermore, multiple daily injections are inconvenient for long-term treatment and may adversely affect the compliance of patients.

Recently, we reported the evaluation of a long-acting formulation of microcapsules on SB-75 acetate (14). For pharmacokinetic studies on SB-75 release from the microcapsules, we developed a highly specific RIA (14). In the present study, we evaluated two recent lots of SB-75 pamoate microgranules. After single injections, both prototype lots (RGS 0001 and RGS 9002) produced an initial "burst" effect within the first 24 hr. Subsequently, lot RGS 0001 showed a rapid decline from peak values, and the release leveled off about the 14th day; this low level was maintained during the rest of the experiment. For lot RGS 9002, after the initial peak, decline in SB-75 levels was more gradual. The peptide concentrations remained relatively stable for 3 weeks and showed a small second peak during the fourth week that was followed by a gradual decline in serum levels of the analog. This pattern of release was similar to that shown by microcapsules of SB-75 acetate lot RGS 0001 (14). However, the duration of release appeared longer with the SB-75 pamoate microgranules. Previously, we showed that the pamoate salt of the somatostatin analog RC-160 in microcapsule formulations exhibited a protracted release as compared with the acetate form (8). Because the primary high release from the microcapsules and/or microgranules ensures a rapid elevated level of antagonist, the initial "burst effect" can be beneficial. Our results show that after a single injection with these sustained-delivery systems, there is a rapid and sustained inhibition of LH and steroid secretion, which is then maintained for prolonged periods by a relatively smaller, but continuous, release of the analog from the microcapsules or microgranules. During the period of SB-75 administration, testosterone levels were depressed. Both lots showed suppression of testosterone to castration levels on day 1 after the injection. Lot RGS 0001 maintained lower testosterone levels, as compared with controls for only 1 week, whereas lot RGS 9002 could decrease testosterone for >42 days. The continuous suppression of testosterone in serum and the decrease in ventral prostate weight throughout the course of the treatment showed that the serum levels of SB-75 released from these microgranules were effective. The doses of SB-75 microgranules used in these experiments were designed primarily for pharmacokinetic studies and were relatively low. Previously, we demonstrated in Copenhagen × Fisher F_1 rats bearing R 3327H Dunning rat prostate tumors that doses of SB-75 microcapsules twice as high as those used in this study greatly inhibited sex steroid-dependent organs (E. Korkut, L.B., K.G., and A.V.S., unpublished results).

The development of LH-RH antagonists free of side effects and the availability of highly efficacious sustained-delivery formulations of these antagonists may allow new approaches for treating sex hormone-dependent cancers and for preventing gonadal damage inflicted by chemotherapy, as well as for therapy of conditions such as endometriosis, leiomyomas, and precocious puberty.

‡Gonzalez-Barcena, D., Vadillo-Buenfill, M., Garcia-Porcel, E., Mares, G., Bajusz, S., Comaru-Schally, A. M. & Schally, A. V., 71st Annual Meeting of the Endocrine Society, June 21–24, 1989, Seattle.

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