Effect of FK506 on Functional Recovery After Facial Nerve Injury in the Rat

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Objective: To examine the effect of the immunosuppressive agent FK506 on the rate of functional recovery of the rat facial nerve after crush injury.

Methods: Forty rats underwent facial nerve crush injury and were randomly assigned to 4 experimental groups: isotonic sodium chloride solution control, FK binding protein 52 (FKBP-52) antibody control, FK506, and FK506 and FKBP-52 antibody. Rats underwent daily recovery testing from postoperative day 9 until postoperative day 21 by videotaping 3 validated variables in this model: blink reflex return, vibrissial fibrillation loss, and return of vibrissial sweeping symmetry.

Results: FK506-treated animals demonstrated improved recovery in all 3 variables compared with control animals. The FK506 and FKBP-52 antibody group demonstrated improved recovery of only the return of the blink reflex.

Conclusions: FK506 accelerated functional recovery of facial nerve function after crush injury. Neuroregeneration was inhibited by FKBP-52 antibody in the rat midface but not the upper face. FK506 may be a viable adjuvant treatment for facial nerve neurapraxic injury.

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Facial nerve injury is a common clinical disorder with serious consequences. The impact of facial nerve injury includes not only loss of aesthetic facial balance but also functional problems, including incomplete eye closure, nasal valve obstruction, oral incompetence, and difficulty with communication via facial expression. Despite its clinical importance, relatively few studies have examined functional recovery of facial movement after facial nerve injury in an animal model. Recently, our laboratory used a simple semi-quantitative method to characterize functional recovery after facial nerve crush injury in a rat model by examining return of blink reflex, loss of vibrissial fibrillations, and return of vibrissial sweeping symmetry.

Several pharmacologic agents have been investigated to determine their possible neuroregenerative effects. FK506, or tacrolimus, is a potent immunosuppressant used to prevent acute and chronic graft rejection after solid organ transplantation. Studies performed during the past decade have demonstrated that FK506 in subimmunosuppressive doses exhibits neuroprotective and neuroregenerative properties. This finding has stimulated interest in characterizing the neurophysiologic effect of FK506 with various nerve injury models. The objective of this study was to examine the effect of FK506 on the rate of functional recovery of the rat facial nerve after crush injury. Functional recovery rate was also examined in animals that received a monoclonal antibody to FK binding protein 52 (FKBP-52), a known FK506 receptor, to further characterize the neuroregenerative mechanism. We hypothesized that FK506 would accelerate facial nerve recovery after crush injury and therefore might ultimately be clinically useful in the management of facial nerve injuries.

Methods

Experimental Design

Forty male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Massachusetts) that weighed 200 to 250 g were randomized to 1 of 4 experimental groups (n=10 rats per group). Group 1 received phosphate-buffered isotonic sodium chloride solution, group 2 received FKBP-52 antibody, group 3 received FK506, and group 4 received FK506 and FKBP-52 antibody. Groups 1 and 2 served as controls for the effects of FK506 and the antibody, respectively. Animals in each group received daily subcutaneous injections of the assigned compounds from postoperative day (POD) 2 to POD 21. Rats were weighed twice weekly to adjust drug doses and assess health. Animals in each group received left facial nerve
crush injury on POD 0. Beginning on POD 9, rats in each group underwent functional recovery testing on a daily basis until POD 21, the expected recovery time frame.6 Animals were killed on POD 21. All animals were cared for under the approved animal care protocol in accordance with all Massachusetts Eye and Ear Infirmary guidelines and regulations.

PREPARATION AND ADMINISTRATION OF COMPOUNDS

FK506 (LC Laboratories, Woburn, Massachusetts) was dissolved in a solution of 20% polyethoxylated castor oil (Cre-mophor; BioChemika, Fluka, Switzerland) and 80% ethanol (Sigma-Aldrich, St Louis, Missouri) to achieve a stock concentration of 10 mg/mL. This stock concentration was diluted in a solution composed of 25% de-ionized water and 75% propylene glycol to a concentration of 2 mg/mL. FK506 was administered to rats in groups 3 and 4 by daily subcutaneous injections at 2 mg/kg per day. The FKBP-52 (also known as FKBP-59 or heat shock protein 56) monoclonal antibody (Stressgen Bioreagents, Victoria, British Columbia) was obtained at 1.0 mg/mL and diluted with phosphate-buffered isotonic sodium chloride solution to 3 stock concentrations of 0.00125, 0.0125, and 0.03125 mg/mL. The FKBP-52 antibody was administered to rats in groups 2 and 4 by daily subcutaneous injections at 1 µg/kg per day (n=3 rats per group), 10 µg/kg per day (n=3 rats per group), and 25 µg/kg per day (n=4 rats per group).

SURGICAL PROCEDURE

Rats were anesthetized with an intramuscular injection of 75 mg/kg of ketamine hydrochloride (Fort Dodge Animal Health, Fort Dodge, Iowa) and 0.5 mg/kg of medetomidine hydrochloride (Orion Corporation, Espoo, Finland). The left infraauricular area was shaved and steriley prepared. The left facial nerve was approached through an infra-auricular incision in each animal by the same investigator (C.Y.). Using the operating microscope, the investigator identified the main trunk of the facial nerve as it emerged anteriorly to the posterior belly of the digastric muscle. The common trunk was electrically stimulated with a nerve stimulator (Montgomery Nerve Stimulator; Boston Medical Products, Westford, Massachusetts) at a setting of 1 mV to verify all hemifacial movement. The nerve was then crushed for 30 seconds using a jeweler’s microforceps, and the crush injury was repeated for an additional 30 seconds in the same location. The loss of electrical conductivity was verified with the nerve stimulator at a setting of 2 mV. The wound was then closed in a single layer with an absorbable suture, and the anesthetic was reversed with a subcutaneous injection of 0.05 mg/kg of atipamezole hydrochloride.

FUNCTIONAL RECOVERY ANALYSIS

Animals underwent daily facial nerve testing according to previously described methods beginning on POD 9.6 Briefly, rats were sedated with 0.5 mg/kg of medetomidine hydrochloride and placed into a body harness on a black, nonreflecting surface. The face of the rat was centered in a circle defined by a 12-in fluorescent light bulb ring for optimal lighting conditions. Videotaping was performed with 2 digital video cameras (Canon GL2; Canon USA, Inc, Lake Success, New York): the first camera was mounted directly above the animal for an optimal view of both vibrissial pads simultaneously, and the second camera was mounted at a 45° angle to the left eye for an optimal view of the blink reflex. The blink reflex was tested first by placing a drop of water into each control eye (right eye) to confirm the presence of the blink reflex under sedation. After verification of right eye blink, a drop of water was placed into the experimental eye (left eye) and videotaping was performed for 3 consecutive attempts (Figure 1). Videotaping of left vibrissial pad fibrillations, if present, was then performed for 10 seconds. After a reversing injection of 0.05 mg/kg of atipamezole hydrochloride was administered, the volitional vibrissial movements were videotaped on the rats’ emergence from sedation for vibrissial sweeping symmetry between the experimental (left) and control (right) sides (Figure 2).

SCORING OF FACIAL FUNCTION

All videotapes were imported into Adobe Premier software (version 6.0; Adobe Systems, Inc, San Jose, California), and analysis of each variable was performed by the same blinded investigator (C.Y.).

Return of Blink Reflex

Frame-by-frame analysis of the 3 consecutive left eye blinks was followed by export of 3 still images of maximal eye opening and maximal eye closing to Adobe Photoshop (version 5.5; Adobe Systems, Inc). Once each eye was corrected to the hori-

Figure 1. Eye movement in an animal with complete return of facial nerve function. A, Maximal eye opening. B, Maximal eye closing.
horizontal plane, measurements of the distance between the upper and lower eyelids at the point halfway between the 2 palpebral fissures were recorded. Mean values of maximal eye opening and maximal eye closure were obtained during the 3 consecutive blinks. A blink score was calculated as follows: 1−(mean maximal eye closure/mean maximal eye opening). Therefore, a blink score of 0 indicated no eye closure, a score of 1 indicated complete eye closure, and decimal values indicated the relative percentage of eye blink.

Loss of Vibrissial Fibrillations

In sedated animals, the presence of fibrillation-related movement of the left vibrissae was given a fibrillation score of 0. Complete absence of vibrissial fibrillations was given a score of 1, indicating full reinnervation of midface branches. When only some vibrissae appeared to be fibrillating, a decimal rank was assigned corresponding to the percentage of vibrissae with loss of fibrillations. Therefore, a fibrillation score of 0.5 indicated that approximately 50% of vibrissae appeared to have loss of fibrillations. The mean fibrillation score for each rat was obtained by averaging the score from each observer.

Return of Sweeping Symmetry

Symmetry was scored by performing frame-by-frame analysis of volitional vibrissial movements during 1 continuous sweep with export of still images of maximal vibrissae protraction and maximal vibrissae retraction to Adobe Photoshop. Each rat’s position was corrected to the vertical plane before analysis. The angle between the horizontal axis and the position of the same individual vibrissae at maximal protraction and maximal retraction was recorded for the control side and the experimental side. The vibrissial sweeping score was calculated as follows:

\[
\frac{(Retraction \ [exp] − Protraction \ [exp]) \times (Protraction \ [cont] − Retraction \ [cont])}{(Protraction \ [cont] − Retraction \ [cont])},
\]

where exp indicates the experimental side and cont indicates the control side. Therefore, a vibrissial sweeping score of 0 indicated complete asymmetry (no movement of vibrissae on the affected side), a score of 1 indicated symmetric vibrissial sweeping, and decimal values indicated the relative percentage of vibrissial sweeping symmetry between the experimental and control sides.

STATISTICAL ANALYSIS

A mean blink score was calculated for each of the 4 groups at each POD by averaging the blink scores of the 10 rats in each cohort at a given POD. The mean fibrillation scores and mean vibrissial sweeping scores were similarly determined for each of the 4 groups at each POD. The 2-tailed t test was used for statistical comparisons between each group at a given POD. Statistical significance was set at \( P < .05 \).

RESULTS

One animal from the isotonic sodium chloride solution control group died on the day of surgery secondary to anesthetic complications. No other intraoperative complications occurred. Postoperatively, no increased morbidity was noted in the drug- or antibody-treated animals, and no systemic or local wound infections developed. Postoperative weight gain was not significantly different among any of the groups. Additionally, no statistically significant differences were found among comparisons of the various antibody dilutions at each POD for groups 2 and 4.

RETURN OF BLINK REFLEX

Return of the blink reflex (blink score ≥ 0.9) was observed by POD 19 in the isotonic sodium chloride solution control group (n=9). Return of function followed a sigmoid curve, with rapid recovery between PODs 13 and 16 and a plateau of recovery by POD 19. The second control group of animals, which received the antibody alone (n=10), demonstrated similar findings to the isotonic sodium chloride solution control group; no significant differences were found between the 2 groups at any postoperative time point.

FK506-treated animals showed earlier return of the blink reflex throughout most of the postoperative course. In the FK506 group (n=10), rapid recovery was seen to occur earlier (between PODs 9 and 13), with near com-
complete return of the blink reflex by POD 16 (Figure 3). The blink score in the FK506 group was significantly greater than in the isotonic sodium chloride solution control group (P < .05) at all time points before plateau, with the exception of PODs 10 and 15. For example, at POD 13, accelerated recovery of the blink reflex was seen because the FK506 group blink score was significantly greater than that of the isotonic sodium chloride solution control group (mean ± SD, 0.73 ± 0.26 and 0.29 ± 0.08, respectively; P < .001).

FK506 and FKBP-52 antibody–treated animals (n = 10) demonstrated a similar pattern of functional recovery to the FK506 group (Figure 4). Rapid recovery occurred between PODs 10 and 13, with near complete return of the blink reflex by POD 17. No statistically significant difference was seen between the FK506 and FKBP-52 antibody group and the FK506 group except at the earliest time points of PODs 9 and 10.

Loss of vibrissial fibrillations, corresponding to reinnervation of the rat midface, followed a sigmoidal course over time, similar to the return of blink reflex. In the isotonic sodium chloride solution control group (n = 9), rapid loss of fibrillations occurred between PODs 13 and 17, with near complete loss by POD 19. The FKBP-52 antibody control group (n = 10) demonstrated no statistically significant difference compared with the isotonic sodium chloride solution control group at any time point except POD 14.

FK506-treated animals (n = 10) showed faster loss of vibrissial fibrillations than both control groups at all postoperative time points before plateau of recovery. This greater loss of fibrillations in the FK506 group compared with the isotonic sodium chloride solution control group occurred between PODs 9 and 16 and reached statistical significance (P < .05) at each of these time points (Figure 5A). Near complete loss of vibrissal fibrillations occurred earlier at POD 15 in the FK506 group compared with the isotonic sodium chloride solution control group (mean ± SD, 0.91 ± 0.06 and 0.62 ± 0.14, respectively; P < .001). No further statistically significant differences between the drug and control groups were seen after the fibrillations disappeared.

FK506 and FKBP-52 antibody–treated animals (n = 10) showed loss of vibrissial fibrillations similar to antibody control animals during all postoperative time points (Figure 5B). No statistically significant difference was seen between the FK506 and FKBP-52 antibody group and the antibody control group at any given postoperative time point. The FK506 and FKBP-52 antibody group demonstrated statistically slower loss of fibrillations compared with the FK506 group from PODs 9 through 14 (P < .05). For example, at POD 12, the FK506 and FKBP-52 antibody fibrillation score was significantly less than the FK506 fibrillation score (mean ± SD, 0.20 ± 0.06 and 0.42 ± 0.05, respectively; P < .001).

Figure 3. Return of blink reflex. A, Recovery of facial function over time for the FK506 group (n = 10) and the isotonic sodium chloride solution (ISCS) control group (n = 9). B, The FK506 group achieves faster return to complete recovery (>90%) of the blink reflex compared with ISCS control animals. Error bars indicate 2-tailed standard deviation. * P < .05.

Figure 4. Recovery of facial function over time for the FK506 group (n = 10) and FK506 and FK binding protein 52 (FKBP-52) antibody group (n = 10), as measured by return of the blink reflex. Error bars indicate 2-tailed standard deviation.

LOSS OF VIBRISSIAL FIBRILLATIONS
RETURN OF SWEEPING SYMMETRY

The return of sweeping symmetry between the operated on and control sides followed a predictable sigmoidal pattern that paralleled the return of blink reflex and loss of vibrissial fibrillations. In the isotonic sodium chloride solution control group (n=9), rapid recovery of symmetrical sweeping occurred between PODs 12 and 16, with a plateau of sweeping symmetry achieved by POD 18. The second control group, animals treated with the antibody alone (n=10), showed a near identical functional recovery to the isotonic sodium chloride solution control group, and no statistical significance was demonstrated at any postoperative time point.

The FK506 group (n=10) demonstrated the highest sweeping symmetry scores at all time points. Additionally, the FK506 group showed earlier rapid recovery of symmetrical sweeping, which occurred between PODs 10 and 13, with a plateau by POD 16 (Figure 6). This faster recovery from injury in the FK506-treated group was significantly different (P<.05) from the isotonic sodium chloride solution control group from PODs 10 through 15 and at POD 17 (mean±SD, 0.96±0.05 and 0.88±0.09, respectively; P=.02).

The FK506 and FKBP-52 antibody group (n=10) had a return of sweeping symmetry pattern that resembled the antibody control group. No statistically significant difference was found between the FK506 and FKBP-52 antibody group and the antibody control group at any postoperative time point. The FK506 and FKBP-52 antibody group demonstrated a statistically slower return of sweeping symmetry compared with the FK506 group (P<.05) at POD 10 (mean±SD, 0.02±0.03 and 0.09±0.08, respectively; P=.01) and from PODs 12 through 18.

COMMENT

FK506 is a powerful immunosuppressant used to prevent graft rejection after organ transplantation. Although FK506 has been well characterized as an immunosuppressive agent, multiple studies have suggested a role in neuroprotection and neuroregeneration. In the central nervous system, FK506 has been shown to protect midbrain dopaminergic neurons in a rodent model of Parkinson disease and exhibits neuroprotective actions in an experimental rodent model of stroke. Additionally, FK506 has also been shown to promote neurite outgrowth in dorsal root sensory ganglia and improve neurologic functional recovery after spinal cord injury in the rat.

Several investigators have examined the neuroregenerative effects of FK506 within the peripheral nervous system. Lee et al11 and Jost et al12 have shown that FK506 accelerates peripheral nerve regeneration in a rat model after sciatic nerve crush injury and posterior tibial nerve transection with immediate epineurial reapproximation. Studies13,14 have also demonstrated the ability of FK506 to rescue posterior tibial nerve allografts in a rat model and to enhance peripheral nerve regeneration in ulnar nerve autografts and allografts in inbred swine. To date, however, few studies have examined the neuroregenerative role of FK506 in an animal cranial nerve model. Diaz et al15 used a rabbit facial nerve model and showed that topical application of FK506 to a transected nerve at the time of repair using entubulation neurorrhaphy resulted in improved regeneration. In their study, histomorphometric analysis of the FK506 group demonstrated preservation of both the nerve cross-sectional area and nerve myelination distal to the transection injury. More recently, Ontanilla et al16 studied the effects of FK506-treated nerve allografts in a monkey facial nerve model. Although the FK506-treated superficial fascial nerve allografts had decreased regeneration compared with nontreated nerve autografts, the authors showed that the FK506-treated allografts stimulated axonal collateralization, resulting in a similar activation of facial nerve muscles. Our group was interested in determining whether the purported neuroregenerative effect of FK506 was present in a model of a rat cranial nerve, namely, the facial nerve.
Investigators have attempted to characterize the central neurologic effects that result from facial nerve injury and to describe the changes to peripheral axons and facial muscle fibers. Few studies have focused on the functional recovery of facial movement after facial nerve injury in an animal model. These studies include measurements of mouse vibrissal movement and analysis of videographic recordings of rat vibrissal movement after facial nerve injury. We recently developed an effective model of rodent facial nerve injury and recovery. Our prior investigation demonstrated that functional recovery of an injured rat facial nerve could be measured by analysis of videographic recordings of 3 recovery variables: return of blink reflex, loss of vibrissal fibrillations, and return of vibrissal sweeping symmetry.

In the present investigation, a randomized study was performed to examine the effect of FK506 on functional recovery of the rat facial nerve after crush injury. All 3 variables of rat facial nerve function, return of blink reflex, loss of vibrissal fibrillations, and return of vibrissal sweeping symmetry, demonstrated a similar pattern of recovery in the isotonic sodium chloride solution control group. The rate of facial nerve recovery followed a sigmoidal pattern in which it started near POD 9, then encountered a period of rapid recovery, with near complete recovery achieved by POD 19. These results are consistent with the rate of facial nerve recovery noted in our previous study. Furthermore, the cohort that received the antibody alone served as an effective second control for the effect of the antibody on the rat because no significant difference was found between the isotonic sodium chloride solution control and the antibody group at any time point in all 3 recovery variables, with the exception of the loss of fibrillations on POD 14, an isolated anomaly.

In this study, FK506 clearly accelerated the neuroregenerative capacity of injured facial nerves. The cohort treated with FK506 achieved faster functional recovery in all 3 studied recovery variables of facial nerve function compared with the isotonic sodium chloride solution control group. Analysis revealed that the higher recovery scores observed in the FK506 cohort were significantly different (P < .05) from the isotonic sodium chloride solution control group for all 3 recovery variables between PODs 9 and 17, with few exceptions. In general, no statistically significant difference was observed between the FK506 cohort and the isotonic sodium chloride solution control group after POD 17 because both groups experienced essentially complete recovery. In summary, the data can be appreciated as a shift of the sigmoid recovery curve to the left toward earlier time points in the animals treated with FK506.

Improved facial nerve recovery after crush injury in rats that received FK506 is consistent with results from prior investigations that used crush injury in lower extremity peripheral nerve models. These studies of sciatic nerve crush injury illustrated that FK506-treated rats had larger mean axonal areas according to morphometric analysis, axons were further advanced toward muscle targets, and restoration of the blood-nerve barrier occurred earlier. It is known that FK506 exerts its immunosuppressive effect by binding to a receptor protein, FKBP-12, which prevents calcineurin from dephosphorylating the transcription factor NFAT. This action blocks movement of NFAT to the cell nucleus and decreases synthesis of interleukin 2, which leads to an overall inhibition of the T-cell immune response. In contrast to the well-characterized immunosuppressive cellular pathway, multiple pathways by which FK506 may exert its neuroregenerative effect have been postulated. These pathways include interactions between FK506 and FKBP-12 and interactions between FK506 and other FKBPs. Prior studies have shown that FK506–FKBP-12 interaction may lead to neuroregenerative effects through increased neuronal expression of a growth cone–associated protein, GAP-43, inactivation of neuronal nitric oxide synthase, or changes to other calcineurin targets.

Gold et al demonstrated in vitro that FK506 maintained its neurotrophic activity in the absence of FKBP-12 binding in hippocampal cell cultures from FKBP-12 knockout mice. This study postulated that the key binding protein FKBP-52 (also known as FKBP-59 or heat shock protein 50) was responsible for the neurotrophic action of FK506 and demonstrated in vitro that the addition of a monoclonal antibody to FKBP-52 completely prevented the neurotrophic action of FK506 in cultured human neuroblastoma cells. FKBP-52, together with heat shock protein 90, is known to be a component of a subclass of steroid receptor complexes. In our present study, we examined the in vivo effect of a monoclonal antibody to FKBP-52 to determine whether the neuroregenerative effect of FK506 could be inhibited in a facial nerve recovery model.

Several explanations exist for the variable effect of FKBP-52 antibody on FK506-treated rats in this investigation. First, inherent neurobiological differences in regenerative capacity may exist among different branches of the facial nerve. The return of blink reflex, which measures functional recovery of the upper division of the rat facial nerve, appears resistant to the addition of FKBP-52 antibody to FK506-treated animals. However, the return of vibrissal sweeping symmetry and loss of vibrissal fibrillations, which are measures of functional recovery of the midface facial nerve branches, appear to be sensitive...
to the addition of FKBP-52 antibody to FK506-treated animals. This factor may indicate a variable sensitivity to FKBP-52 antibody in the different distributions of facial nerve fibers. Alternatively, other FK506 neuroregenerative mechanisms, such as calcineurin-dependent FK506–FKBP-12 interactions, may play a more important role in the recovery of upper division facial nerve fibers. Second, the return of blink reflex may be under dual motor control. Recently, we observed that the retractor bulbi muscle may play a more important role in the return of blink reflex than previously thought. If the return of blink reflex were controlled by both facial nerve–generated motor signals and non–facial nerve–generated retractor bulbi muscle function, it would not be surprising that FK506-induced recovery of upper division facial nerve function would not be completed inhibited by FKBP-52 antibody. A third plausible explanation is the possibility of trigeminal neoneuroregeneration. In our experiment, the lack of inhibition by FKBP-52 antibody on FK506-accelerated recovery of the rat upper face may suggest that interactions between dormant connections of the trigeminal nerve branches and branches of the facial nerve upper division play an important role after crush injury to the rat facial nerve. In the future, further work with a number of animals, investigation of FK506–FKBP-12 interactions in this rodent model, and study of the dual motor control theory for the blink reflex will help clarify the effect of FKBP-52 antibody on FK506-treated rats.

In summary, the present investigation illustrates that administration of FK506 results in faster facial nerve functional recovery after crush injury in both the upper division and midface branches of the rat facial nerve. Furthermore, the FK506-induced neuroregeneration can be inhibited by administration of FKBP-52 antibody in the midface facial nerve branches but not the upper division of the facial nerve. Low-dose FK506 may be a viable adjuvant treatment to facial nerve injury. This treatment would be a welcome addition to the limited armamentarium that currently exists to treat severe neurapraxic injury to the facial nerve. Even in cases of facial nerve injury in which complete functional recovery is expected over time, the ability to accelerate recovery time would significantly improve patient treatment and minimize the emotional and functional impact of longstanding facial paralysis.

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Author Contributions: Dr Hadlock had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Yeh and Hadlock. Acquisition of data: Yeh, Bowers, and Hadlock. Analysis and interpretation of data: Yeh, Bowers, and Hadlock. Drafting of the manuscript: Yeh and Hadlock. Critical revision of the manuscript for important intellectual content: Yeh, Bowers, and Hadlock. Statistical analysis: Yeh and Hadlock. Obtained funding: Hadlock. Administrative, technical, and material support: Yeh, Bowers, and Hadlock. Study supervision: Hadlock.

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