versible G_0 phase, from which they can be removed by means of injury (*18*). Thus far, no manipulation of the pituitary (or hormones controlled by it) has been found to initiate corneal endothelial growth or to modify it where stimulated by injury (*8*). Since both populations of cells are bathed only by the aqueous humor and since just one cell type is responsive to injections of somatomedin C, these results suggest that proliferation in the two tissues is controlled by different mechanisms.

The lens epithelium, in contrast, is exquisitely sensitive to pituitary manipulation. The work reported here is the first, to our knowledge, in which a purified somatotropin-dependent mitogen has been found to restore growth that has been completely stopped in vivo.

The lens epithelial cell of the hypophysectomized frog has been shown to reside in a "deep" G_0 state. Such a cell requires about 50 percent longer (72 as opposed to 48 hours) to reach DNA synthesis after introduction of the organ into a suitable medium in vitro (*5*). Yet, somatomedin C is able to transpose such cells through the entire generative cycle. In Balb/c 3T3 cells, somatomedin C has been interpreted by Stiles *et al.* (*19*) to act as a progression factor—a factor whose presence is required after the "decision" to evacuate G_0 has been taken. Balb/c 3T3 cells must first be rendered competent (for example, by wounding or exposure to platelet-derived growth factor) before progression factors such as somatomedin C permit DNA synthesis to occur (*20*). The present studies do not clarify whether, in frog lens epithelial cells, somatomedin C works in concert with other endogenous non-growth hormone-dependent competence factors to permit DNA synthesis to occur or whether, in this tissue, induction of competence is unneccessary for the action of somatomedin C.

Three pituitary hormones (growth hormone, frog prolactin, and thyrotropin) can promote proliferation in the lenses of hypophysis-deprived frogs in vivo but not in vitro (*4, 7, 21*). All three enhance the synthesis of somatomedin C in the mammalian liver (the last, via the thyroid gland) (*22*). Bovine and ovine prolactin do not stimulate growth in the frog lens (*4*); this may be due to species specificity, since White and Nicoll (*23*) were unable to detect receptors to ovine prolactin in bullfrog liver. However we have shown (*23*) that hGH does not bind to frog hepatocytes. Insulin, insulinlike growth factors I and II, and epidermal growth factor stimulate growth in rabbit lens epithelium in vitro (*24*). Insulin exerts this effect on the epithelium of the organ-cultured frog lens (*25*). The effect of somatomedin C on the frog lens in vitro remains to be investigated.

HOWARD ROTHSTEIN
Kresge Eye Institute of Wayne State University, Detroit, Michigan 48201
JUDSON J. VAN WYK
Department of Pediatrics, Endocrinology Division, University of North Carolina, Chapel Hill 27514
JOHN H. HAYDEN
SHELDON R. GORDON
Kresge Eye Institute of Wayne State University, Detroit
ALLAN WEINSIEDER
Department of Anatomy, Wayne State University School of Medicine, Detroit

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23 October 1979

Alcoholic Rhabdomyolysis: An Experimental Model in the Rat

Abstract. *The main features of alcoholic rhabdomyolysis—skeletal muscle necrosis, marked elevation of serum creatine phosphokinase, and myoglobinuria—were produced in rats by a combination of relatively prolonged (2 to 4 weeks) exposure to ethanol and a brief period of food deprivation. This observation suggests that fasting may similarly trigger muscle injury during binge drinking in man. The effect of fasting is in part related to an increase in blood alcohol due to reduced alcohol clearance and in part caused by a fasting-induced potentiation of the toxic effects of high concentrations of alcohol on skeletal muscle.*

Acute alcoholic myopathy, or alcoholic rhabdomyolysis, was first described by Hed and his co-workers and has since been increasingly recognized as a complication of severe alcoholism (*1*). It is manifested by rapidly evolving muscle destruction, with weakness, muscle pain, sudden and marked elevation of serum creatine phosphokinase (CPK), myoglobinuria, and histological evidence of muscle fiber necrosis, degeneration, and regeneration (*2*).

The pathogenesis of alcoholic rhabdomyolysis is unknown. It has not been reproduced experimentally (*3*), although administration of alcohol to human volunteers (*4*) or experimental rats (*5*) has been reported to cause mild elevation of

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serum CPK and slight ultrastructural or histochemical changes in muscle. The fact that this syndrome occurs clinically in the setting of binge drinking (*1*), where exposure to high concentrations of alcohol is commonly associated with poor nutrition (*6*), suggested to us that the combination of alcohol exposure and nutritional stress may lead to muscle injury in this condition. We here report the production of experimental alcoholic rhabdomyolysis (EAR) in rats by withdrawal of food during chronic exposure to ethanol.

Female Sprague-Dawley rats (180 to 230 g) were maintained in Plexiglas alcohol vapor chambers for 3 weeks (*7*). The concentration of ethanol vapor in the chamber was adjusted to give blood alcohol concentrations usually in the range of 100 to 300 mg per 100 ml, as verified by daily measurements of blood samples from one or more animals. The animals were tube-fed once a day, either as a supplement to ad lib commercial rat chow to ensure adequate nutritional intake or as the sole nonalcohol nutritional source. Liquid diets, either without vitamins and minerals or fully supplemented with both (*8*), were used as indicated. Regardless of the diet, weight loss in the experimental animals averaged 16 percent of the initial weight after 3 weeks of alcohol exposure. Control rats were fed restricted amounts of rat chow in order to limit the body weight comparably. During fasting, food was withheld for periods of 1 to 3 days. Additional control animals were deprived of food for up to 7 days. In the experimental group, alcohol exposure was continued during fasting. Water was freely available to experimental and control animals at all times.

Serum CPK levels were measured by the method of Rosalki (*9*, p. 789). Blood alcohol was determined spectrophotometrically (*9*, p. 1499). Serum sodium and potassium concentrations were measured by flame photometry (*10*), and serum phosphate was measured colorimetrically (*11*).

During 3 weeks of continuous alcohol exposure, animals given a complete diet (rat chow or vitamin- and mineral-supplemented liquid diet) had no significant elevation of serum concentrations ($P > .2$) (Fig. 1). However, when rats that had been exposed to alcohol continuously for 3 weeks were food-deprived for 1 to 3 days while alcohol administration continued, the serum CPK rose sharply. Figure 1 shows the mean of the highest CPK concentrations reached during fasting. In many instances the rise of CPK occurred within 24 hours after the first missed feeding. Sixty percent of the food-deprived, alcohol-treated animals developed CPK values greater than 2 standard deviations above the mean of food-deprived controls, and 11 percent had CPK values greater than ten times the control mean.

Pigmenturia was detected in 14 of 45 animals in which the urine was examined. It was presumptively identified as myoglobinuria on the basis of a positive urine *o*-tolidine reaction, in the absence of microscopic hematuria and in the presence of a clear serum (*12*). The identification was verified in five of five instances where sufficient urine was available by a positive immunochemical reaction for myoglobin (*13*).

Histological examination of extensor digitorum longus, soleus, or tibialis anterior muscles of ten animals with markedly elevated serum CPK (range, 727 to

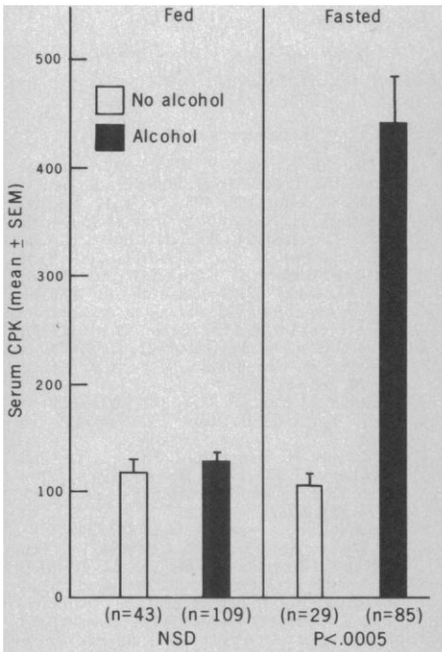

Fig. 1 (left). Serum CPK (mean ± SEM) in alcohol-treated and control (no alcohol) rats in the fed and fasted states; NSD, not significantly different. Diets were as follows: for the fed, alcohol-treated rats, ad lib rat chow (91 animals) or complete liquid diet (18 animals); for the control rats, rat chow. During fasting, alcohol administration was continued in the alcohol-treated animals. The combination of alcohol treatment and fasting resulted in a marked increase in the mean CPK.

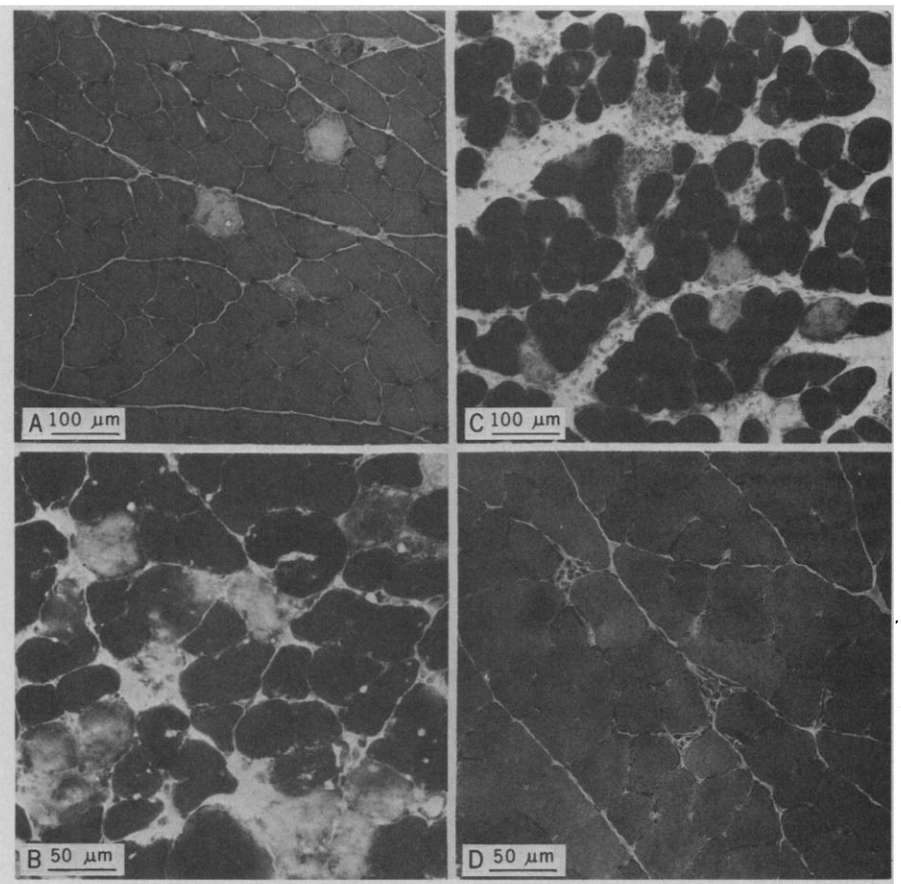

Fig. 2 (right). Histology of experimental alcoholic rhabdomyolysis (EAR). (A) Scattered, pale, necrotic fibers in otherwise normal-appearing muscle. (B) Severe EAR with many fibers undergoing floccular degeneration. (C) Many fibers undergoing phagocytosis in severe EAR. (D) Advanced phagocytosis of scattered fibers in otherwise normal muscle.

2758 mIU/ml) revealed typical findings of rhabdomyolysis (Fig. 2). Muscle fibers were pale and swollen, with many showing floccular or vacuolar necrotic changes, cellular infiltration, and phagocytosis. The degree ranged from mild, with rare necrotic fibers in muscle that otherwise looked normal, to massive, with large confluent areas of necrosis. The muscle histology in fed, alcohol-treated rats was normal.

These findings were not attributable to fasting alone. Control rats that were deprived of food for up to 7 days but not exposed to alcohol did not develop elevated serum CPK (Fig. 1) or pigmenturia, and the muscle histology in these animals was normal. Mineral and vitamin deprivation alone did not lead to rhabdomyolysis in alcohol-treated rats. In experiments in which tube-feeding was the sole source of nonalcoholic calories, serum CPK values at 2 to 3 weeks of alcohol exposure were not significantly different in animals fed mineral- and vitamin-supplemented diets (CPK ± SEM = 125 ± 9.1, n = 18, where SEM is the standard error of the mean) compared to mineral-free (137 ± 18, n = 11) and vitamin- and mineral-free (138 ± 23, n = 12) diets.

These results suggest that rats tolerate blood alcohol concentrations in the range of 100 to 300 mg per 100 ml for weeks with little change in the serum CPK, even with the additional stress of vitamin and mineral deficiency. However, short-term food deprivation during continued alcohol exposure results in elevated serum CPK in most animals, with the full clinical syndrome of alcoholic rhabdomyolysis, including myoglobinuria and muscle necrosis, in some.

Food deprivation is known to reduce the hepatic oxidation of alcohol, resulting in higher blood concentrations in fasted than in fed animals at comparable alcohol exposures (*14*). We found that, after 36 hours of food deprivation, the blood alcohol concentration was 39 percent higher in fasted animals than in fed, alcohol-treated animals. In order to evaluate the possibility that this rise in blood alcohol might account for the muscle injury seen with starvation, we analyzed serum CPK as a function of simultaneously determined blood alcohol concentrations in fed and fasted alcohol-treated rats (Fig. 3). These results indicated that serum CPK rises significantly as a function of increasing alcohol concentration in both groups. However, the mean CPK was higher at each blood alcohol concentration in fasted animals than in fed, alcohol-treated animals. Moreover, extreme elevations of serum CPK (greater than ten times normal) with marked myoglobinuria were produced only in the fasted group. These results suggest that alcohol has a direct toxic effect on skeletal muscle but that additional factors must act to potentiate the toxic effect of high blood alcohol concentrations during periods of food deprivation.

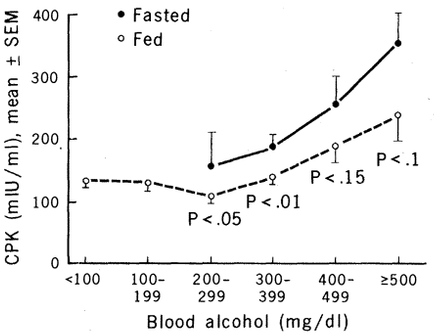

Fig. 3. Serum CPK (mean ± SEM) as a function of simultaneously determined blood alcohol in fed and fasted rats. Samples were obtained in the fed rats after 2 to 3 weeks of alcohol exposure (126 samples, 72 rats) and in the fasted rats within 24 to 72 hours of the start of fasting after 2 to 3 weeks of alcohol exposure (96 samples, 49 rats). The P values show the differences between fed and fasted animals. At each blood alcohol range, the fasted animals had higher CPK concentrations than the fed animals.

Other factors that have been implicated in the pathogenesis of alcoholic rhabdomyolysis include hypokalemia (*15*) and hypophosphatemia (*16*). To investigate the possible role of these electrolyte abnormalities in experimental alcoholic rhabdomyolysis, we measured sodium, potassium, and phosphate concentrations in serum obtained 12 to 24 hours before the onset of myoglobinuria in fasted, alcohol-treated animals. Serum sodium and phosphate concentrations were normal, whereas serum potassium was lower in both fed, alcohol-treated (4.0 ± 0.4 meq/liter) and fasted, alcohol-treated animals (3.9 ± 0.2 meq/liter) compared to fasted controls (4.9 ± 0.4 meq/liter). Such mild hypokalemia is not associated with skeletal muscle pathology in the rat (*10*). Further, mild hypokalemia was present in both fed and fasted alcohol-treated animals, whereas rhabdomyolysis occurred only in the fasted animals. Thus it seems unlikely that this slight hypokalemia can account for muscle injury in EAR.

These studies indicate that the cardinal laboratory and histological features of alcoholic rhabdomyolysis—marked elevation of serum CPK, myoglobinuria, and muscle fiber necrosis—can be reproduced in rats by a combination of alcohol exposure and food deprivation. This combination mimics the events of the alcoholic binge that typically precede human alcoholic myopathy (*17*) and suggests that fasting during an alcoholic debauch may trigger muscle injury in the clinical situation. The enhancing effect of food deprivation in EAR is due in part to the fasting-induced increase in blood alcohol concentration caused by reduced alcohol clearance. The high alcohol concentration appears to exert a toxic effect on muscle cells, as indicated by the correlation between blood alcohol concentrations and serum CPK in the alcohol-treated rats. This observation is consistent with earlier reports that have suggested that alcohol may directly injure skeletal (*4, 18*) or cardiac (*19*) muscle. However, our study further indicates that additional and as yet unidentified factors related to food deprivation may potentiate the myotoxic effect of alcohol, since the CPK values of fasted rats were higher than those of the fed rats at the same blood alcohol concentrations. Further studies of this model may fully elucidate the biochemical mechanisms of alcohol-induced muscle injury.

RONALD G. HALLER
DANIEL B. DRACHMAN
Department of Neurology,
Johns Hopkins School of Medicine,
Baltimore, Maryland 21205

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30 November 1979

Regression of Tumors in Guinea Pigs After Treatment with Synthetic Muramyl Dipeptides and Trehalose Dimycolate

Abstract. *A high incidence of tumor regression was observed in guinea pigs bearing transplantable, line-10 hepatocellular carcinomas when synthetic muramyl dipeptides combined with trehalose dimycolate in oil-in-water emulsions were injected directly into the tumors. These compounds are promising candidates to replace viable bacillus Calmette-Guérin in cancer immunotherapy in humans and animals.*

Approximately 8 years ago, a report (*1*) appeared describing the use of viable *Mycobacterium bovis*, strain bacillus Calmette-Guérin (BCG), to treat dermal and metastatic tumors in guinea pigs. This model system subsequently proved useful for evaluating immunopotentiating substances with antitumor activity for use in the treatment of human cancer (*2-4*). Here we describe the use of synthetic, water-soluble muramyl dipeptides (also termed adjuvant dipeptides) in combination with trehalose dimycolate (isolated from mycobacteria) to replace viable mycobacteria in this immunotherapy model system.

Tumors in humans (*5, 6*) and animals (*2*) have been treated by injecting live BCG directly into the growing tumors. However, such treatment may produce slowly healing ulcers, protracted fever, hepatitis, and occasional anaphylactic reactions (*2, 5-8*). Because of these undesirable and sometimes life-threatening side effects, it has been a goal of many investigators to replace viable BCG with defined, nontoxic components isolated from mycobacteria. Such replacement would also assist investigators to study the biological processes of tumor regression after immunotherapy. Considerable progress was made in this direction when Ribi and co-workers found that oil-in-water emulsions containing either cell walls of mycobacteria (*9*) or cell wall skeletons combined with trehalose dimycolate (*10*) were effective in curing tumors in guinea pigs. These nonviable preparations have been used successfully in clinical trials for treatment of spontaneous tumors in humans (*3*) and animals (*11*).

The component of mycobacterial cell wall preparations that is responsible for tumor regression is not known, but the synthetic compound *N*-acetylmuramyl-L-alanyl-D-isoglutamine represented a reasonable candidate. This unit was found to be the minimal structural entity that can replace mycobacterial whole cells in Freund's complete adjuvant and permit antibody stimulation and delayed hypersensitivity reactions to a variety of antigens (*12*). Covalently lipid-bound muramyl dipeptides suppressed growth of tumors in mice (*13*) and promoted regression of tumors in guinea pigs (*4*). On the basis of these observations and with the hope of finding a potent muramyl dipeptide, we investigated the antitumor activity of certain synthetic analogs of *N*-acetylmuramyl-L-alanyl-D-isoglutamine.

Ten different muramyl dipeptides (150 μg of each) (*14*) were tested alone and in combination with trehalose dimycolate (150 μg). Test materials were admixed in mineral oil and then suspended in 0.2 percent Tween 80 in phosphate-buffered saline to form oil-in-water emulsions (*15*). The final concentration of oil was 0.75 percent by volume. Test emulsions (0.4 ml) were injected directly into established, line-10 hepatocellular carcinomas 6 days after intradermal transplantation of 10^6 tumor cells. The tumors were 8 to 11 mm in diameter and had metastasized to regional draining lymph nodes (*2*). Surviving animals received another transplantation of 10^6 line-10 cells 3 months after treatment and were observed for an additional month. All of the animals rejected this challenge.

In the absence of trehalose dimycolate, none of the muramyl dipeptides caused regression of tumors in treated animals (*16*). Trehalose dimycolate was inactive when tested alone. The tumor-regressive activity of the muramyl dipeptides combined with trehalose dimycolate was greatest for *N*-acetyl-4,6-di-*O*-octanoylmuramyl-L-valyl-D-isoglutamine and *N*-acetyldesmethylmuramyl-L-α-aminobutyryl-D-isoglutamine (Table 1). Subsequent dose-response studies indicated that the latter is the more effective compound on a per microgram basis (*17*). Although all of the tested muramyl dipeptides except *N*-acetylmuramyl-D-alanyl-D-isoglutamine showed adjuvant activity (*18*), three of these compounds in combination with trehalose dimycolate did not have significant antitumor activity. Substitution of an *N*-glycolyldesmethylmuramic acid moiety for an *N*-acetylmuramic acid group or substitution of L-serine, L-valine, or L-α-aminobutyric acid for the L-alanyl moiety significantly increased the antitumor activity of the muramyl dipeptides combined with trehalose dimycolate.

Table 1. Tumor regression after treatment. Values are numbers of guinea pigs cured of dermal and metastatic tumors over numbers of animals treated. Data shown are pooled from two separate experiments. No cures were observed in animals treated with any muramyl dipeptide in the absence of trehalose dimycolate (*16*).

Synthetic muramyl dipeptide (150 μg) tested with trehalose dimycolate (150 μg)	Observed tumor regression
N-Acetylmuramyl-L-alanyl-D-isoglutamine	1/17
N-Acetylmuramyl-D-alanyl-D-isoglutamine	0/9
N-Acetyl-4,6-di-*O*-octanoylmuramyl-L-alanyl-D-isoglutamine	1/9
N-Acetyldesmethylmuramyl-L-alanyl-D-isoglutamine*	2/19
N-Acetylmuramyl-L-threonyl-D-isoglutamine	3/9
N-Acetylmuramyl-L-seryl-D-isoglutamine	10/17†
N-Acetyldesmethylmuramyl-L-valyl-D-isoglutamine*	10/17†
N-Glycolyldesmethylmuramyl-L-alanyl-D-isoglutamine	7/9†
N-Acetyl-4,6-di-*O*-octanoylmuramyl-L-valyl-D-isoglutamine	16/18†
N-Acetyldesmethylmuramyl-L-α-aminobutyryl-D-isoglutamine*	17/18†
Trehalose dimycolate alone (control)	0/17
Emulsion of oil, Tween 80, and phosphate-buffered saline (control)	0/14

*These are trivial names for 2-acetamido-2-deoxy-D-gluco-3-*O*-yl-acetyl dipeptides. †Significantly different from the value for trehalose dimycolate-treated controls (*21*) (chi-squared, 2 × 2 table analyses, $P \leq .05$).

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