motor nerve endings in response to exogenous ACh.

The effect of venomous impairment on the action of injected ACh in the innervated neuromuscular junction was disclosed more directly in three experiments by antidromic monitoring. We found that the motor nerve discharges which ordinarily follow intra-arterial ACh injection (*3*) were uniformly absent. Because this ACh-evoked neural activity arises in the nerve ending, the absence of firing after venom injection is not unexpected in view of the motor nerve terminal destruction described above.

The limited ACh contractile responses that remain after venom poisoning (Fig. 3) likely express the reactivity of the postjunctional membrane to ACh. This dose-response relationship is, we think, an unequivocal quantitative description of postjunctional reactivity to ACh. The destruction of the motor nerve terminals has eliminated the large neural component in this response, and denervation hypersensitivity is not yet present. Furthermore, the post-venom dose-response relationship (Fig. 3) shows that no matter how large the ACh dose (including doses up to 1 mg/kg) the contractile response never exceeds the twitch height, as is regularly the case in the normal system when doses of 50 μg/kg or larger are given. Therefore the much greater responses of the normal muscle must reflect not only the synchronizing influence of the widespread ACh evoked motor nerve terminal discharge but also the repetitive nature of that response. Thus, selective poisoning of motor nerve terminals by spider venom confirms in yet another way, the conclusions reached in earlier studies from this laboratory, wherein it was shown by two distinctly different methods, namely antidromic monitoring (*3*) and subacute denervation (*4*), that the motor nerve terminals are the major site of exogenous ACh action.

Because of the apparently selective and irreversible action of black widow spider venom on motor nerve endings, there is at hand a unique means by which the functional and pharmacologic properties of these important nerve endings may be explored and related to fine structure. However, if the full potential of this reagent is to be realized, it will be necessary to isolate the active principle. This would enable, through dose-response analysis, a full and precise definition of its selectivity at the neuromuscular complex.

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L-Glutaminase: Suppression of Lymphocyte Blastogenic Responses in vitro

Abstract. *Continuous exposure to 0.1 international unit or more of* Escherichia coli L-*glutaminase inhibited responses of human lymphocytes to phytohemagglutinin, streptolysin O, and allogeneic leukocytes. Inhibition was completely reversed by removing the enzyme from the culture or adding* L-*glutamine but not* L-*asparagine. Cytoxicity did not occur.* L-*Glutaminase should be immunosuppressive in vivo.*

In the search for new immunosuppressive agents, the effects of the enzyme L-glutaminase on the in vitro blastogenic responses of human peripheral blood lymphocytes to mitogen stimulation were investigated. It was hypothesized that L-glutaminase, which catalyzes the conversion of L-glutamine to L-glutamic acid, might be immunosuppressive for the following reasons. Glutamine antagonists such as azotomycin are immunosuppressive (*1*). Although L-glutamine is not an essential amino acid, lymphoid tissue contains a small amount of glutamine-synthetase (*2*) and gets its L-glutamine from the blood (*3*). Some cells have a population-dependent requirement for L-glutamine in vitro (*4*). Experimental lymphoid malignancies are susceptible to the antitumor action of L-glutaminase (*5*), and specific enzyme-induced amino acid deficiency is immunosuppressive in the case of L-asparaginase (*6*) which may contain 1 to 3 percent glutaminase activity.

The lymphocyte culture system was selected for initial studies of this enzyme because it has provided significant data on the function of the human lymphoid system (*7*) and has been useful in evaluating the mechanisms of action of immunosuppressive drugs such as azotomycin (*1*) and L-asparaginase (*6*). Cultures were set up according to described methods (*8*). Each tube (13 by 100 mm) contained 4×10^5 lymphocytes, in a medium composed of 0.3 ml of autochthonous serum, 0.7 ml of L-glutamine–free and L-asparagine–free minimal essential medium (Spinner-modified), and 100 units of penicillin and 100 μg of streptomycin per milliliter. Cultures were stimulated with one of the following mitogens: 0.02 ml of phytohemagglutinin (PHA), 0.03 ml of streptolysin O (SLO), or 4×10^5 allogeneic lymphocytes previously irradiated with 5000 rads (WBC) (*9*). *Escherichia coli* L-glutaminase (*9*) was dissolved in saline, kept at 4°C and used within 2 weeks. L-Glutamine was supplied as a 200 m*M* solution, was stored frozen and thawed only once before use. L-Asparagine (*9*) was dissolved in saline, frozen only once, and thawed before use. These cultures were incubated at 37°C for 3, 5, and 7 days, respectively, and then incubated for 2 hours in the presence of 2 μc of tritiated thymidine (specific activity, 1.9 c/mmole). Acid-insoluble radioactivity was measured by liquid-scintillation counting (count/min per 4×10^5 lymphocytes). Slide preparations were also made and the percentages of lymphoblasts in 500 cell counts were recorded.

L-Glutaminase, when present from the beginning of the culture period, was a potent inhibitor of blastogenesis in vitro (Table 1). Doses of 0.01 I.U./ml were significantly inhibitory. Doses of 0.1 I.U./ml inhibited thymidine incorporation approximately 87 to 95 percent

Table 1. Effect of L-glutaminase on lymphocyte blastogenic responses to phytohemagglutinin (PHA), streptolysin O (SLO), and allogeneic leukocytes (WBC) measured by [H³]thymidine incorporation (count/min) or slide counts of the percentage of lymphoblasts. All cultures were done in duplicate, and there were nine sets of cultures for [³H]thymidine studies and one set for blastogenic studies. The *P* values give significance of comparison of indicated value and preceding value. In each case L-glutaminase and mitogen were added at the beginning of the culture period.

L-Glutaminase dose (I.U./ml)	Incorporation of [³H]thymidine: (mean, 10^3 count/min) (*P*)			Blastogenesis (percentage of lymphoblasts)		
	PHA	SLO	WBC	PHA	SLO	WBC
None	30.7 ± 7.7	16.0 ± 13.9	27.3 ± 16.8	89	41	15
0.01	12.7 ± 11.4 (*.001*)	5.2 ± 3.0 (*.05*)	14.1 ± 9.0 (*.02*)	77	16	8
0.05	4.7 ± 3.8 (*.05*)	4.9 ± 4.0 (*.50*)	5.4 ± 4.1 (*.05*)	79	22	6
0.10	1.9 ± 1.1 (*.05*)	2.0 ± 2.9 (*.05*)*	1.9 ± 1.9 (*.05*)	35	7	4
0.50	0.2 ± 0.18 (*.001*)	0.08 ± 0.01 (*.05*)†	0.3 ± 0.3 (*.05*)	8	1	0
1.00	0.08 ± 0.05 (*.05*)	0.06 ± 0.04 (*.80*)	0.08 ± 0.06 (*.20*)	0	0	0

* *P* value compares 0 and 0.10. † *P* value compares 0.05 and 0.5.

while doses of 1.0 I.U./ml inhibited thymidine incorporation by more than 99 percent. The blastogenic responses to PHA, SLO, and WBC were similarly inhibited. The degree of inhibition of the morphological parameters of blastogenesis paralleled the degree of inhibition of thymidine incorporation. L-Glutaminase was equally inhibitory whether added 24 or 48 hours after initiation of the cultures and stimulation with mitogen. However, it was less inhibitory when added after 72 hours, which suggested accumulation of intracellular stores of L-glutamine, increased glutamine-synthetase, or development of alternate pathways in the stimulated cells.

Inhibition of blastogenesis was apparently due to specific depletion of L-glutamine. This was proved by the complete reversal of inhibition when L-glutamine was added at the beginning of the culture period (Table 2). The effects of 0.1 and 0.5 I.U./ml were completely reversed by 2 m*M* L-glutamine. When 20 m*M* L-glutamine was added, inhibition was again noted. This inhibition was subsequently shown to be due to the glutamic acid produced by the enzyme's action on the added L-glutamine (see below). The addition of up to 2 m*M* L-asparagine, in contrast, did not reverse the effects of the enzyme (0.1 or 0.5 I.U./ml), and higher doses could not be used because such doses were themselves toxic to lymphocytes (*6*).

Inhibition of blastogenesis was achieved without cytotoxicity. First, the enzyme could be washed from the cultures after up to 72 hours of continuous exposure, and if both mitogen and L-glutamine were added to the cultures at that point, the subsequent blastogenic responses were completely normal. Second, counts of quantitative viable cells, as judged by trypan blue dye exclusion (*6*), showed that cells continuously exposed to up to 1 I.U. of enzyme per milliliter for up to 72 hours maintained viability identical to the controls.

Because glutamic acid is the product of reaction catalyzed by L-glutaminase and because small doses of L-glutamine corrected inhibition while larger doses of L-glutamine in L-glutaminase treated cultures were inhibitory (Table 2), the effects of L-glutamic acid on lymphocyte blastogenesis were studied. Doses of glutamic acid at 2 m*M* or under did not inhibit blastogenesis (Table 2). Doses at 20 m*M* partially inhibited lymphocyte responses and doses at 100 m*M* abolished them. This inhibition was reversible however, and lymphocyte responses were normal at 96 hours when the acid was washed from the cultures at up to 72 hours of continuous exposure.

These studies indicate that L-glutaminase is a potent, reversible inhibitor of in vitro lymphocyte blastogenic responses. Complete inhibition was observed at 1 I.U./ml, a concentration comparable to that which is well tolerated in mice (*5*). Inhibition was the result of specific depletion of L-glutamine. It was reversed by the addition of L-glutamine, although at concentrations of L-glutamine over 2 m*M*, a return of inhibition was probably caused by the glutamic acid generated by the enzyme's action. Accumulation of inhibitory concentrations of glutamic acid would not be expected in vivo since the serum concentration of L-glutamine is only 0.57 m*M* (*10*).

Extrapolating from these results obtained in vitro suggests that this enzyme would be an immunosuppressive drug in vivo. L-Glutaminase should be most effective when administered repeatedly before the administration of antigen and continuing through the usual proliferative phase of the immune response. The effect of such treatment will depend on whether increased levels of glutamine-synthetase are induced by the depletion of L-glutamine. If such increases occur, potential immunosuppressive potency will be limited. Thus, induction of asparagine-synthetase limits the therapeutic usefulness of L-asparaginase (*11*). Usefulness of the enzyme in vivo might also be limited by the degree of associated toxicity. Neurologic and gastrointestinal toxicity and inhibition of synthesis of coagulation factors have severely limited the usefulness of L-asparaginase in vivo (*12*).

Thus there is now a third potential or actual immunosuppressive enzyme, which, like the others, acts by deaminating an amino acid. The first is L-asparaginase which deaminates a nonessential amino acid (*6*). The second is arginine deaminase which has been shown to be the cause of the inhibition of blastogenesis produced by certain

Table 2. Effects of various additives on PHA-induced blastogenesis in L-glutaminase treated and untreated cultures. Data are the means of three experiments in which response was measured by [H³]thymidine incorporation (count/min). In each case, PHA, L-glutaminase, and the given additive were present throughout the culture period.

Amount added (m*M*)	[³H]Thymidine incorporation (mean, 10^3 count/min) at various doses of L-glutaminase (I.U./ml)		
	0	0.1	0.5
	Glutamine		
0	35.0	2.1	0.4
0.2	44.8	6.1	2.3
2.0	60.2	40.4	36.2
20.0	46.4	18.3	10.3
	Asparagine		
0	26.0	1.2	0.15
1.0	27.6	1.8	0.05
2.0	20.1	3.2	0.46
	Glutamic Acid		
0	21.0		
2.0	17.5		
20.0	3.8		
100.0	0.4		

strains of mycoplasma (*13*). To these we can now add L-glutaminase; our studies indicate that its immunosuppressive properties should be explored in vivo.

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DNA Synthesis and Interaction between Controlled Feeding Schedules and Partial Hepatectomy in Rats

Abstract. *The rate of DNA synthesis has been measured during liver regeneration in rats adapted to a controlled feeding schedule. The results show two different phenomena in the regulation of DNA synthesis. The first is the appearance of a peak of DNA synthesis following the operation itself and independent of the time of the day; the second one is the presence of constant diurnal variations in the rate of DNA synthesis in response to the partial hepatectomy and following the stimulus or stimuli of the controlled feeding schedule.*

The regeneration of rat liver after partial hepatectomy has been extensively studied by many authors since the early experiments performed by Higgins and Anderson in 1931 (*1*). Several reviewers have reported the features of liver regeneration, and the literature is extensive (*2, 3*). As far as DNA biosynthesis is concerned, it is known that it begins after a lag period of about 18 hours and peaks between 20 and 30 hours after partial hepatectomy (*2*). The age of rats (*4*), as well as the percentage of liver excised (*5*), influences the timing of the DNA synthesic period. A second peak of the incorporation rate of thymidine (TdR) into DNA is known to occur between 65 and 72 hours after the operation (*6, 7*). Rhythmic periodicity of mitosis at 24-hour intervals was first observed in regenerating rat liver by Jaffe (*8*), and in unoperated young animals by Jackson (*9*). Halberg and Barnum and their colleagues (*7, 10*) were able to show circadian rhythms in the in vivo labeling of DNA in normal and regenerating rodent liver. In order to conduct a systematic study of the variation in rate of DNA synthesis in rat liver and hepatomas, Potter *et al.* (*11–13*) have developed a "controlled feeding schedule." Under these experimental conditions they found large diurnal variations of the incorporation rate of TdR into DNA in both hepatoma and host liver, using the transplantable Morris hepatoma 7793. Below we report experiments dealing with the diurnal variation of the incorporation rate of TdR into DNA in normal growing liver, and report comparisons with regenerating liver studied in rats adapted to a "controlled feeding schedule."

Male Sprague-Dawley rats obtained from either the Charles River Breeding Laboratories, Inc., the Holtzman Company, or the ARS/Sprague-Dawley Company were used throughout all experiments. Unoperated rats were obtained soon after weaning and were used at about 35 days of age (Fig. 1). Partial hepatectomies were performed on older rats (58 to 62 days old) weighing 230 to 260 g (Fig. 2). The rats were housed on arrival in an air-conditioned windowless room with an inverted and displaced lighting schedule in which lights were on from 8:30 p.m. (20:30) to 8:30 a.m. (8:30) in a 24-hour cycle. The diet contained 30 percent protein (*12*), and food was supplied just before the lights were switched off. Food dishes were removed 8 hours later according to the "8 + 16" feeding schedule developed by Potter *et al.* (*12, 13*). Water was always available. The rats were adapted to this protocol for at least 1 week before being used for the experiments. Partial hepatectomies were performed under ether anesthesia, with removal of the main lobes (66 to 72 percent of the liver was excised) as described by Higgins and Anderson (*1*). The operations were performed at 20:30 ± 30 minutes, at 15:30 ± 30 minutes, or at 11:30 ± 30 minutes, as indicated in Fig. 2, A, B, and C, respectively.

Thymidine-methyl-^{3}H (New England Nuclear Co., specific activity 6.45 c/mmole) was used to evaluate DNA synthesis. At intervals ranging between 15 and 56 hours after the partial hepatectomies, and at different times during the 24-hour day, the rats were injected intraperitoneally with thymidine-methyl-^{3}H (20 μc/100 g of body weight in 0.4 ml of sterile saline) and killed by cervical dislocation exactly 1 hour later. The livers were quickly removed and dropped into cold saline, blotted with absorbent tissue, and homogenized in nine volumes of distilled water with a Polytron homogenizer (*14*). Perchloric acid was added to an aliquot of the homogenate to a final molarity of 0.5. After centrifugation, the supernatant was saved and the residue was washed once with cold 0.5*M* perchloric acid. The supernatants were combined and designated "acid-soluble fraction." DNA in the pellet was separated from RNA as described by Munro and Fleck (*15*) and was assayed by the Ceriotti procedure (*16*) slightly modified (*17*). Radioactivity was measured on a Packard Tri-Carb liquid scintillation spectrometer.

The lower portion of Fig. 1 shows the 1-hour incorporation rate of TdR into DNA in the normal, growing, young rat liver during the course of the day. Rats 35 days old were used for these experiments in order to detect some DNA synthesis which is almost completely abolished in adult rats. The data are expressed as percentage of total uptake (defined as the sum of the radioactivities in the DNA and the acid-soluble fraction at 1 hour after injection). This way of express-