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Short Communications

Uninterrupted protein synthesis is essential for survival in the early stages of carbontetrachloride-induced hepatocellular necrosis in the mouse

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Summary. The fatal syndrome produced by cycloheximide given 6 h after a hepatonecrogenic dose of CCl_4 is due neither to direct toxic synergism between CCl_4 and cycloheximide nor to transient sinusoidal thrombosis. It is suggested that survival in the presence of unknown factors released from dying liver cells requires uninterrupted protein synthesis. The life-saving effect of sterilization of the intestine by antibiotics indicates that the gut flora or its products play a vital role in pathogenesis.
Key words. CCl_4; hepatocellular necrosis; protein synthesis inhibition; Ancrod; antibiotics; gut flora.

A lethal, shock-like syndrome occurs when mice given a hepatonecrogenic dose of CCl_4 are challenged 6 h later (but not if challenged 18 h later) with a dose of 30 µg/g b.wt of cycloheximide[2]. It was shown that heparin protected against these fatalities, leading to the tentative hypothesis that transient centrilobular obstruction by sinusoidal thrombosis led to irreversible ischaemic damage to the midgut[2]. Cycloheximide was thought to act by preventing protein synthesis, suggested by other work[3] to be essential for the physiological release of endogenous anticoagulant heparin.

The present report extends these initial observations, shows that thrombosis plays no essential part in the evolution of this lethal syndrome, and indicates a more complex pathogenesis involving the process of hepatocellular necrosis and the gut flora or its products.

Materials and methods. Male, inbred CBA-strain mice, 20–25 g in weight were used, and allowed free access to food and drink throughout.

Carbon tetrachloride (Analar grade, B.D.H. Ltd, England) was mixed with an equal volume of olive oil (B.P.), and 0.1 ml of the mixture given by s.c. injection. Prior observation confirmed that this dose caused centrilobular hepatic necrosis but no detectable renal tubular necrosis. Cycloheximide (Sigma Chemical Co. Ltd, England) was dissolved in sterile 0.9% aqueos NaCl solution to give a concentration of 30 µg/0.01 ml, and given i.p. as a dose of 30 µg/g b.wt. This dosage gives approximately 90% inhibition of protein synthesis as measured in the mouse liver[4], and is tolerated by all normal mice. Ancrod, 70 U/ml (Armour Pharmaceutical Co. Ltd, England), was diluted with sterile saline to give a concentration of 0.05 U/0.01 ml, and injected i.p. as a dose of 0.05 U/g b.wt. At this dose level, Ancrod produces complete defibrination in the mouse (personal communication from Berk Pharmaceuticals, Ltd, England). Neomycin sulphate and bacitracin (Sigma Chemical Co., Ltd, England), 4.0 g of each were dissolved in 1.0 l of boiled, cooled tap water and the solution brought to pH 4.0 by addition of 1.0 N HCl. This antibiotic solution was substituted for drinking water for 4 full days before start of the relevant experiment as recommended by van der Waaij and Sturm[5], and replaced by tap water 48 h after the start of the experiment.

All injections were given under light ether anesthesia. Moribund mice were killed by cervical dislocation under ether anesthesia; such animals, together with mice dying during the course of the experiments, were necropsied and major organs sampled for routine histological examination.

Statistical analysis of the results was by the Fisher-Irwin exact probability test (two-sides).

Experiment 1. Three groups each of five mice were used. Group A mice were given Ancrod followed immediately by CCl_4; 6 h later the mice received cycloheximide. Group B mice received

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Experiment	Group	Treatments and times	Result Number of dead/moribund mice Total number of mice in group
1	A	Ancrod/CCl_4 (0 h) Cycloheximide (+6 h)	5/5
	B	CCl_4 (0 h) Ancrod (+4 h) Cycloheximide (+6 h)	5/5
	C	Saline/CCl_4 (0 h) Cycloheximide (+6 h)	5/5
2	D	CCl_4 (0 h) Cycloheximide (+6 h) (antibiotics given)	0/8
	E	CCl_4 (0 h) Cycloheximide (+6 h) (no antibiotics)	8/9
3	F	CCl_4 (0 h) CCl_4 (+24 h) Cycloheximide (+30 h)	0/5

Significance of results: group D vs group E, $p = < 0.001$. Group D vs group E + group C, $p < 0.0001$.

CCl_4 followed in 4 h by Ancrod, and 2 h later were given cycloheximide. Group C animals were treated like group A, but sterile saline was substituted for Ancrod.

Experiment 2. Group D (eight mice), was given the antibiotic solution as the only fluid source for 96 h, then challenged with CCl_4 followed 6 h later by cycloheximide. (To determine whether or not antibiotics as used here have an effect on the degree of CCl_4-mediated centrilobular necrosis, two mice (group D_1) were treated as group D, but with omission of cycloheximide. These animals were killed 48 h after CCl_4 injection and their livers fixed for histological examination.)

Group E (nine mice), was treated as group D, but animals were allowed to drink tap water instead of antibiotic solution.

Experiment 3. Five mice (group F) were given CCl_4 in the right flank; 24 h later, the animals received a second dose of CCl_4 in the left flank, followed in 6 h by cycloheximide i.p.

Results. Experimental protocols and results are summarized in the table.

Experiment 1. All mice (groups A, B and C) developed a severe, rapidly progressive illness; all animals were either dead or moribund by 29 h after cycloheximide challenge. Post mortem examination showed a pale liver and dilated, congested small intestine. Histologically, intestinal congestion was confirmed and there was evidence of early necrosis of villous tips. The liver showed centrilobular necrosis and severe vacuolation of surviving periportal hepatocytes. Thus, it is clear that Ancrod confers no protection.

Experiment 2. Antibiotic-pretreated animals (group D) all resisted the otherwise-fatal challenge by CCl_4/cycloheximide without developing a severe clinical illness, whereas, as expected, the majority of the control (group E) mice were dead or moribund 24 h after cycloheximide challenge. Post mortem findings in this group were identical to those in groups A, B and C. One animal in group E developed a moderately severe illness, but recovered and by 48 h was clinically normal.

The two antibiotic-treated mice challenged with CCl_4 and killed 48 h later (group D_1), showed a degree of centrilobular hepatocellular necrosis indistinguishable from that customarily seen in CCl_4-injected mice of this strain which have received no other treatment.

Experiment 3. No fatalities and no serious illness occurred in these mice (group F).

Discussion. It has been clearly shown that heparin treatment is life-saving in mice given a hepatonecrogenic dose of CCl_4, then challenged 6 h later with cycloheximide[2]; this finding led to the tentative suggestion that cycloheximide interfered with endogenous anticoagulation mechanisms[3], allowing transient centrilobular sinusoidal thrombosis to develop and cause irreversible ischemic damage to the midgut. The present results make such a hypothesis untenable. Such postulated thrombosis would require conversion of fibrinogen to fibrin; not only should heparin treatment therefore be effective, but adequate defibrination should also be protective. The ineffectiveness of Ancrod used in a dose known to cause complete defibrination in the mouse, shows that conversion of fibrinogen to fibrin can play no significant part in the evolution of this fatal syndrome. This reasonably explains the failure to detect centrilobular thrombosis by histological examination at various times during development of the fatal syndrome (Parry, unpublished observations).

The clinical features of the fatal syndrome are consistent with a shock-like state, and it was considered remotely possible that endotoxin might play a part in its development. Non-absorbable broad-spectrum antibiotics as used here are known to sterilize effectively the intestinal tract of the mouse[5], and would thus be expected to reduce or abolish the production and absorption of endotoxin. The complete protection conferred by such antibiotic treatment (group D) suggests that the normal gut flora and/or their products play a crucial role in pathogenesis. The beneficial effects of antibiotics cannot be attributed to any protection against the hepatonecrogenic effect of CCl_4, since antibiotic-treated mice (group D_1) developed the expected degree of liver necrosis.

Heparin is known to have a beneficial effect in experimental endotoxic shock, possibly acting at more than one point in the complex series of events following administration of endotoxin[6]. This may explain the demonstrated, non-anticoagulant protective effect of heparin in the CCl_4/cycloheximide syndrome[2].

How the combined effects of CCl_4 and cycloheximide bring about the intolerance of normal gut flora must at present remain conjectural. However, it is clear that a direct toxic synergism between CCl_4 and cycloheximide can play no part, since following an initial dose of CCl_4, a second dose given 24 h later, followed in 6 h by cycloheximide does not result in serious illness or fatality (group F). Such considerations, together with the fact that no fatalities occur when CCl_4 and cycloheximide administration are separated by 18 h[2], suggests that it is the early stage of the process of hepatocellular necrosis itself rather than the loss of hepatocytes which is the crucial factor. One working hypothesis is that survival in the presence of materials leaking from the dying liver cells in the early stages of necrosis requires uninterrupted protein synthesis. Cycloheximide inhibition of protein synthesis allows these materials to induce fatal susceptibility to the gut flora or its products.

Thus, a hitherto unrecognized factor, namely uninterrupted protein synthesis, is shown to be essential for survival in the early stages of hepatocellular necrosis. The nature of the essential protein(s), and the way they protect against the fatal effects of the normal gut flora is clearly of as much interest as is the identity of the postulated factor(s) emanating from the dying liver cells, and which are presumed to be responsible for initiating the fatal sequence of events.

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