Chemical Histologic and Immunologic Responses in Rats to CCl_4 By Different Routes of Administration.* (30535)

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No systematic study has been made on the comparative hepatotoxicity of carbon tetrachloride (CCl_4) administered by different routes. Anti-liver antibodies are not generally associated with hepatotoxicants unless cirrhosis is induced by moderate, repetitive doses over a period of many days(1). This is in contrast to the recognized role which immune responses play in the pathogenesis of drug-induced hepatitis. In the present study, liver histology, liver lipids and hydroxyproline content, and anti-liver antibodies were utilized to study the effects of a single small dose of CCl_4 given by various routes of administration.

Methods. Male albino rats of the Sprague-Dawley strain weighing 350 g or more were used in all experiments. Water and Rockland rat diet were given *ad libitum*. Rats were divided into 4 groups: normal, controls and those given a single 0.25 ml dose of CCl_4 by inhalation, subcutaneous injection or gastric intubation. Inhalation doses were calculated from average minute volumes, the assumption of 50% retention, and were administered at 6000 ppm using a dual syringe feeder and a stainless steel inhalation chamber. Rats were sacrificed by decapitation 7 days after the exposure.

Part of the tissue was quick-frozen, stored at $-10°C$ and the remainder fixed in 10% formalin. Tissue sections for microscopy were stained with Hematoxylin-Eosin, Periodic acid-Schiff (PAS), and Oil Red-O for each animal. Each slide was graded on a double-blind basis for lipidosis, fibrosis, necrosis, congestion and PAS positive material content.

Liver lipids were measured by the Folch method(2). Liver hydroxyproline was determined by the technique of Leach(3).

Boyden's tanned erythrocyte hemagglutination test was adopted for the Takatsy-type microtiter technique(4). Ouchterlony gel precipitation tests were performed using the Feinberg technique(5). Intracutaneous skin tests using liver extracts were read at 20 minutes, 24 and 48 hours. Liver antigens were prepared by extracting the minced tissues with phosphate buffered isotonic saline pH 6.5. Four kinds of liver were extracted: Normal rat liver and liver from rats given a single 0.25 ml subcutaneous injection of CCl_4, 4 hours, 24 hours or 5 days before.

Results. Table I indicates mean values of liver lipids and hydroxyproline content. Liver lipids in the gastric group were elevated above all the other groups ($p<0.05$). The other groups did not differ significantly from each other. The liver hydroxyproline contents of the rats which received CCl_4 by the gastric or subcutaneous route were elevated above the normal and inhalation groups ($p<0.01$) but were not themselves different. Liver hydroxyproline content was similar in the normal and inhalation groups.

Table II summarizes the degree of lipid degeneration observed in the livers of rats. If one compares the groups showing $>2+$ changes, then the gastric group showed more lipidosis than the normal, inhalation or subcutaneous groups ($p<0.001$, <0.001, and

TABLE I. Liver Lipid and Hydroxyproline Content in Rats Given CCl_4 by Different Routes.

Exp groups	No. of rats	% Liver lipids (dry wt)	No. of rats	Liver hydroxyproline*
Normal	23	11.7 ± 3.4†	21	3.7 ± 2.6†
Inhalation CCl_4	19	10.8 ± 6.4	19	3.7 ± 2.2
Subcutaneous CCl_4	8	15.6 ± 2.5	11	4.9 ± 1.5
Gastric CCl_4	13	23.0 ± 6.4	13	4.9 ± 1.0

* Hydroxyproline expressed as $\mu g/2$ mg of dry defatted liver.
† Mean ± S.D.

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TABLE II. Lipidosis in Livers of Rats Given CCl_4 by Different Routes.

Exp groups	No. of rats	Degree of lipidosis 4+	3+	2+	1+	0
Normal	23				2	21
Inhalation CCl_4	19				8	11
Subcutaneous CCl_4	9		1	4	1	3
Gastric CCl_4	13	7	4	2		

TABLE III. Liver Necrosis in Rats Given CCl_4 by Different Routes.

Exp groups	No. of rats	Extent of necrosis 4+	3+	2+	1+	0
Normal	23					23
Inhalation CCl_4	19	2	2	2	7	6
Subcutaneous CCl_4	9		1		2	6
Gastric CCl_4	13	9	4			

<0.05) respectively. Similarly there was more lipid degeneration seen in the subcutaneous group than in the normal or inhalation groups ($p<0.005$). There was no significant histologic difference between the liver lipidosis in the inhalation and normal groups.

The most extensive necrosis followed gastric administration (Table III). Using any degree of necrosis as a criterion, the gastric ($p<0.01$), subcutaneous ($p\ 0.025>p>0.01$), and inhalation ($p<0.005$) groups differed from the normals. While the gastric group differed from the subcutaneous ($p<0.005$), the inhalation group did not differ from the gastric or subcutaneous.

Congestive changes were similar for all. Glycogen depletion, estimated by a decrease in PAS positive material in the liver sections, showed no decrease in the normal or inhalation groups but there was approximately one half as much PAS positive material in the livers of animals from the subcutaneous and gastric groups.

Table IV presents the results of the hemagglutination tests for anti-liver extract antibodies. There was no significant difference in the low occurrence of positive hemagglutinations between groups regardless of the antigen extract used. All skin tests and Ouchterlony gel precipitin tests were negative regardless of the antigen preparation used. More animals were added to this phase of the study because of the expected low yield of positive hemagglutinations.

Discussion. The liver lipid values by group are in good agreement with the histologic features of lipidosis observed. Even after 7 days the gastric route of CCl_4 appears to be more lipidogenic than the other routes of administration employed. This is interpreted to mean that the acuteness of the liver injury is greater *via* the gastric route, producing greater inhibition of liver protein synthesis, more cellular necrosis and a decrease in lipid release as lipoprotein. It suggests that dilutional factors, opportunities of CCl_4 excretion, metabolism, and deposition in lipid depots attenuate the acuteness of CCl_4 impact and decrease the cumulative dose to the liver(6).

Rats given CCl_4 by the gastric and subcutaneous routes have significantly increased liver hydroxyproline content when measured 7 days after exposure, which suggests that the stimulus for fibroelastic proliferation is increased in these groups. It is not remarkable that increases in liver hydroxyproline content were observed in absence of histologic evidence of fibrosis in these liver sections. Menon's work showed that liver hydroxyproline content is a superior quantitative index of connective tissue than its histologic demonstration because it is more sensitive and circumvents the problem of non-uniform

TABLE IV. Incidence of Positive Hemagglutinations Against Liver Extracts by Experimental Groups.

Exp groups	No. of rats tested	Antigen extract: Normal liver	Liver post-CCl_4 exposure: 4 hr	24 hr	5 days
Normal	26	1	0	0	0
Inhalation CCl_4	32	2	2	0	0
Subcutaneous CCl_4	16	0	0	1	0
Gastric CCl_4	13	0	1	1	2

deposition(7). There is no correlation between the presence of necrosis and the increases in hydroxyproline content by group. Unfortunately, the lack of correlation between the groups showing increased liver hydroxyproline content and histological evidence of necrosis does not clarify the position of necrosis as an antecedent pathogenic requirement for fibroelastic proliferation in the damaged liver(8).

Acute liver injury shows lipidosis, glycogen depletion and congestion. These features are seen most commonly in the livers of rats in the gastric group, least in the inhalation group, with the subcutaneous group intermediate.

The overall incidence of anti-liver hemagglutinations in these groups is low and agrees with the reports of other workers(9). Isoimmune responses to organ injury are usually of the primary type, with antibodies appearing transiently after 10 to 14 days. These animals were sacrificed 7 days post-CCl_4 injury. Such antibodies as were observed could be the result of a secondary immune response in an occasional animal with prior liver injury. It is also possible that these positive serum hemagglutinations represent the persistence of liver-released immunoglobulins such as found by Wier following liver damage induced by hepatotoxic agents including CCl_4. There is no reliable association between the animals with positive liver hemagglutinations, degree of necrosis, or elevation of liver hydroxyproline content. The negative skin tests observed signify the failure to induce the hypersensitivity phenomenon. The negative Ouchterlony precipitins demonstrate the greater sensitivity of the hemagglutination technique. Positive anti-kidney hemagglutinations were present in 2 of the rats with concomitant anti-liver hemagglutinations. These rats belonged to the inhalation group where the impact of CCl_4 on the kidney might be greater. Absorption of the anti-liver positive serums with rat kidney removed this reaction while preserving the anti-liver hemagglutination but in lower titer (1:64 to 1:8). This limited observation does not indicate whether the immunoglobulins removed were kidney specific or also possessed liver specificity as the work of Pressman suggests(10). In each instance where a serum gave a positive hemagglutination, an extract of the animal's own liver gave a similar result. This means that the animal was responding to his own liver constituents. Some rats gave hemagglutination reactions to the different liver antigen extracts. These differences are not significant and indicate that no antigenic changes are induced in the livers of rats given CCl_4 under these conditions. This agrees with the precipitin studies on this point by Frank(11).

Summary. Rats given a single, small dose of CCl_4 by gastric intubation showed a significant elevation of liver lipids and liver hydroxyproline when sacrificed after 7 days. Rats which received a comparable dose by subcutaneous injection showed only elevated hydroxyproline levels. When the same dose was administered by inhalation neither liver lipids nor hydroxyproline content were increased. The incidence of microscopic lipidoses was greatest in the gastric and subcutaneous groups, while the incidence of necrosis was greater in the gastric and inhalation groups than in the subcutaneous group. A few immune responses were demonstrated by anti-liver hemagglutinations but none by Ouchterlony gel precipitin or skin tests.

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