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Received November 16, 1966. P.S.E.B.M., 1967, v124.

Chemical, Histologic and Immunologic Responses in Rats to CCl_4 II. Relative Influence of Choline Deficiency.* (31898)

VERNON N. DODSON, DOROTHY F. KETCHUM, AND ROBERTA DE MOTT FRIBERG
(Introduced by H. J. Magnuson)
Department of Industrial Health, School of Public Heath, Institute of Industrial Health, University of Michigan, Ann Arbor

Previous reports have indicated that 2 types of immunoglobulins appear in the blood of rats following carbon tetrachloride (CCl_4) poisoning: those which are not organ-specific, released by hepatocytes within a few hours after exposure and persist for one week; and, the anti-liver antibodies which appear two weeks following exposure, remain for several months and occasionally cross-react with kidney antigens(1,2). Previous work suggested a relationship between these latter antibodies and the liver lipids and liver hydroxyproline levels in non-cirrhotic rats(3). This study reports the results of immunologic assay for isologous anti-liver antibodies and chemical analyses of the livers of rats which had been fed a choline deficient diet, poisoned by CCl_4 or both.

Methods. Male albino rats of the Sprague-Dawley strain weighing 350 g or more were used throughout. Rats were divided into 4 groups: normal controls fed a regular diet, those fed a choline deficient diet 2 or 4 weeks prior to CCl_4 exposure and those given a regular diet but exposed to CCl_4. All food and water was supplied *ad libitum*. The Standard Rockland Rat Diet was used for the regular diet while the choline deficient diet was prepared by Nutritional Biochemical Co. Inhalation doses of CCl_4 were calculated from average minute respiratory volumes, with the assumption that 50% of the CCl_4 was retained. The drug was administered at 6000 ppm using a dual syringe feeder and a stainless steel inhalation chamber equipped with an air flow greater than 20 liters per minute. Rats were sacrificed by decapitation 14 days after their CCl_4 exposure, while the unexposed animals were killed at comparable intervals.

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.

Liver lipids were measured by the Folch method(4). Liver hydroxyproline was determined by the technique of Leach(5).

Boyden's tanned erythrocyte hemagglutination test was adapted for the Takatsy-type microtiter technique(6). Ouchterlony gel precipitation tests were performed using the Feinberg method(7). 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 0.1 M 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 shows the content of lipids and hydroxyproline in the livers of rats by experimental groups. These data were analyzed for variance by arranging the experimental groups into columns and rows according to the scheme in Table II.

Groups 9, 5 and 6 were significantly different at the 0.5% level for liver content of lipids and hydroxyproline and the lipid/hydroxyproline ratio (L/OHP) displaying in-

*This study was supported in part by Grant OH-00116 from USPHS.

TABLE I. Liver Lipid and Hydroxyproline Content in Rats Fed a Choline Deficient Diet, Given CCl_4 by Inhalation, or Both.

Group	No. of rats	Duration of CDD	Inhalation dose CCl_4 in ml	% liver lipids (dry wt)	Liver hydroxyproline*	Ratio liver lipids/ hydroxyproline
1	4	4 weeks	.25	64.4†	6.3	10.1
2	3	4 "	.5	54.4	6.1	9.0
3	4	6 "	.25	62.8	5.7	8.7
4	2	6 "	.5	61.6	4.9	15.4
5	4	4 "	0	38.4	4.2	9.5
6	4	6 "	0	75.3	5.9	12.3
7	4	0	.25	7.3	3.3	2.2
8	6	0	.5	10.3	4.7	2.9
9	21	0	0	11.6	3.7	3.2

* Hydroxyproline expressed as μg/2 mg of dry defatted liver.
† Mean values.

creased concentrations of these substances with longer periods on the choline deficient diet. A similar trend is observed between Groups 7, 1 and 3, ($P<0.005$) despite the 0.25 ml dose of CCl_4. Groups 8, 2 and 4 only differed at the 1% level for the lipid and OHP content when the 0.5 ml dose of CCl_4 was used. Data was insufficient for an analysis of the L/OHP ratio between Groups 2 and 4.

TABLE II. Scheme for Analysis on Computer by Experimental Groups.

		Duration of choline deficient diet (CDD)		
		None	4 weeks	6 weeks
CCl_4 Dose (ml)	0	9*	5	6
	.25	7	1	3
	.5	8	2	4

* Group number (see Table I).

The analysis of groups within each column as well as group aggregates by row indicated that no significant differences were induced by CCl_4 in the lipids or hydroxyproline content in the livers of these rats.

As seen in Table III, positive hemagglutinations were most frequent with antigen extracts prepared from the livers of rats exposed to CCl_4 24 hours prior to sacrifice. None were detected in the 5 day post-CCl_4 rat livers, and a few were found using normal or 4 hour post-CCl_4 rat livers. Except for the one rat in Group 6 which reacted to the normal liver extract, all reactions observed using the 4 hour post-CCl_4 and normal liver extracts occurred in Groups 1 through 4, *i.e.*, among those rats which received a choline deficient diet and CCl_4.

Table IV compares mean values of the concentrations of lipids and hydroxyproline in the livers of rats *vs* the presence or absence of serum anti-liver hemagglutinins. Rats whose serums gave positive hemagglutinations using the 24 hour post-CCl_4 liver antigen extract had significantly higher liver lipid values and lower liver lipid-hydroxyproline ratios than

TABLE III. Prevalence of Positive Hemagglutinations Against Liver Extracts by Experimental Groups.

Exp groups		No. of rats tested	Normal liver	Antigen extract: Liver post-CCl_4 exposure 4 hr	24 hr	5 days
1	(CDD-4, CCl_4-.25)*	4	1	0	2	0
2	(CDD-4, CCl_4-.5)	3	0	1	2	0
3	(CDD-6, CCl_4-.25)	4	0	1	1	0
4	(CDD-6, CCl_4-.5)	2	1	1	2	0
5	(CDD-4)	4	0	0	2	0
6	(CDD-6)	4	1	0	2	0
7	(CCl_4-.25)	4	0	0	0	0
8	(CCl_4-.5)	6	0	0	0	0
9	(Normal control)	21	0	0	0	0

* CDD-4 = Choline deficient diet for a total of 4 wk of which 2 were prior to a dose of carbon tetrachloride of .25 ml (CCl_4-.25).

TABLE IV. A Comparison of Liver Lipids and Hydroxyproline Content in 50 Rats with Negative and Positive Hemagglutinations.

	% Liver lipids (dry wt)	Liver hydroxyproline*	Ratio of liver lipid/ hydroxyproline
Hemagglutination			
Positive	63.26 ± 16.37†	5.20 ± .98†	12.85 ± 1.71†
Negative	21.29 ± 21.28†	4.46 ± 1.26†	4.40 ± 3.26†
F-ratio comparison	36.74	3.93	56.37
Significance	.1%	Not significant	.1%

* Hydroxyproline expressed as μg/2 mg of dry defatted liver.
† Mean ± S.D.

TABLE V. Comparison of Amount of Lipids in Livers of Rats with Their Anti-Liver Hemagglutination Status.

	% Liver lipids (dry wt)					
	30–39	40–49	50–59	60–69	70–79	80–89
Hemagglutination						
Positive	2*	0	1	4	2	2
Negative	1	3	1	1	2	1

* No. of rats in each category.

those that did not hemagglutinate, while there was no real difference in the hydroxyproline content of the liver between these groups.

Table V shows the distribution of lipid content *vs* the results of anti-liver hemagglutination reactions.

None of the rats developed histologic changes of cirrhosis. Animals with elevated liver lipid values also showed a parallel increase of lipid in their tissue sections.

In all instances, the Ouchterlony gel reactions between the various extracts of liver and the sera of the animals were negative. All of the intracutaneous tests performed using a wide range of dilutions of the various liver extracts were non-reactive in all animals.

Discussion. In this study, choline deficiency primarily was responsible for the elevated liver lipids observed. The degree of lipidosis was related to the duration of the choline deficiency. Six weeks of choline deficiency produced a mean liver lipid concentration of 75.3% in Group 6, compared to 38.4% in Group 5 which sustained the deficiency for only 4 weeks and 11.6% for the normal controls in Group 9. Probably because the rats had 14 days during which to recover from their CCl_4 exposures, none of the groups that received only CCl_4 reflected any significant alterations in the fat content of their livers. Even Groups 1 through 4 displayed no evidence of CCl_4 enhancement of lipidosis 14 days after its administration despite the choline deficient diet. In fact, CCl_4 given to choline deficient rats seems to have attenuated differences expected in liver lipids as the result of the duration of this deficiency in Group 1 *vs* 3, and 2 *vs* 4.

The accumulation of fat within the hepatocytes is the result of many factors acting in concert. In the case of CCl_4, it appears to depend mostly upon inhibited protein synthesis failing to provide adequate lipoproteins for lipotropism. Choline deficiency, on the other hand, seems to generate lipidosis not only by reducing lecithin formation, but also reducing long chain fatty acid oxidation. In this study, CCl_4 seems to have enhanced the lipidosis induced by choline deficiency when lipid concentrations were relatively low, *e.g.*, 38.4% in Group 5 which received only 4 weeks of the deficient diet, compared to 64.4% and 54.4% in Groups 1 and 2 respectively which received CCl_4 in addition to the 4 week choline deficient diet. Any augmentation of the choline deficiency-induced lipidosis by CCl_4 was not discernible when the fat concentrations were high as evidenced by similar fat concentrations in Groups 3, 4 and 6. It is possible that the magnitude of

such facilitation was too small to be detected at these levels by the methods employed or that the lipidogenic potential for hepatocytes was reached for that time interval. It seems unlikely that CCl_4 exerted no effect.

While the data in Table IV indicate a significant correlation between positive hemagglutinations and elevated liver lipid content, it does not relate this fact to specific animals. Table V compares these parameters and shows that among the animals with positive hemagglutinations, none had liver lipid values below 32% and more were above 60%, indicating a tendency for this relationship to occur regardless of hemagglutination status. If one views the degree of lipidosis as a measure of liver injury by the two methods employed in this study, then positive hemagglutinations begin to show a trend of increasing prevalence when liver lipid concentrations exceed 50%, which might suggest that as lipoprotein and phospholipid factors are inhibited by CCl_4 or choline deficiency respectively, anti-liver factors emerge. The data in Table III indicate that the immune response seems related primarily to the role played by choline deficiency rather than CCl_4 intoxication since no hemagglutinations were observed in the rats given only CCl_4 while 50% of the choline deficient rats exhibited positive hemagglutinations. Furthermore, CCl_4 did not change this frequency when administered to the rats already on the choline deficient diet.

The greater sensitivity of hemagglutination compared with precipitin methods might account for the apparent immunologic differences. While a similar explanation could be responsible for the difference in reactivity observed between the skin tests and the hemagglutinations, this appears less likely than an interpretation based upon differences in the type of hypersensitivity involved, *i.e.*, a cell-mediated globulins(3). As was pointed out previously, liver damage by hepatotoxicants is not usually immunologically mediated, hence the paucity of positive hemagglutinations in the rats given CCl_4(3). The presence of hemagglutinations is quite different in the choline deficient groups where lipotropism is interfered with at the phospholipid synthesis level, suggesting that factors which influence phosphorylation and its precursor pathways result in the appearance of anti-liver substances in the serum.

Summary. Rats were exposed to two different dose levels of CCl_4 by inhalation while on a normal diet or one deficient in choline for 2 or 4 weeks. The lipid and hydroxyproline content and the histology of the livers were studied. Anti-liver factors were determined by hemagglutination and precipitin methods and skin tests utilizing extracts prepared from normal and CCl_4-exposed rats. Choline deficiency produced the greatest lipidosis in the liver and was associated with a high prevalence of anti-liver serum factors by hemagglutination. Under the conditions of these experiments, CCl_4 did little to influence these parameters.

The authors wish to express their appreciation to Ian Mitchell, Jean Pence and Lucas DeVries for technical assistance and to Dr. John A. Jacquez for assistance in the analysis by computer.

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Received November 18, 1966. P.S.E.B.M., 1967, v124.