EFFECT OF REPEATED ADMINISTRATIONS OF CARBON TETRACHLORIDE ON THE VITAMIN B₁₂ CONTENT IN SERUM AND LIVER OF RABBITS

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Previously it was reported that a single administration of carbon tetrachloride in rabbits induced a marked elevation of the serum vitamin B₁₂ (B₁₂) levels and a reduction of the liver B₁₂ levels, and that the two had a close relationship to each other regarding time and quantity and also to the development of the liver damages provoked by CCl₄ (1, 2). The changes in the B₁₂ content of the serum and liver recovered after complete reparation of the liver damages. Similarly, in patients with acute hepatitis there was a marked elevation of the serum B₁₂ levels, which gradually returned to a normal range concomitantly with the recovery of the illness (3). In cirrhosis of the liver, however, persistent high levels of the serum B₁₂ were present (3, 4). The present study has been conducted to examine whether such a persistent high level of the serum B₁₂ can be produced by repeated administration of CCl₄ in rabbits.

EXPERIMENTAL

Materials and Methods

Animals — Rabbits weighing 2.0 to 3.0 kg were used, which had been kept on the diet of our laboratory for at least a week before being used in the experiment.

Administration of Carbon Tetrachloride — Rabbits were repeatedly injected subcutaneously with a 20 per cent solution of CCl₄ in olive oil in a dose of 0.5 ml per kg body weight each time.

Vitamin B₁₂ Determination — Microbiological assay with *Lactobacillus leichmannii* ATCC 4797 was used. The procedure for the assay was described previously (1, 2). The bloods and liver tissues were obtained and treated in the same manner as in the previous reports (1, 2). The determination of alkali-stable fraction of B₁₂ activity in the serum and liver was found to be unnecessary as stated previously (1, 2).

Histopathological Observations of the Liver Tissues — The liver tissues were also examined histopathologically. They were stained with hematoxylin and

eosin, fat was stained with Sudan III, glycogen by the method of Hotchkiss (5), and ribonucleic acid with pyronine-methyl green applying ribonuclease digestion (6).

RESULTS

1. Changes in Serum B_{12} Levels of Rabbits Repeatedly Injected with CCl_4

It was shown in a previous report (2) that by a single injection of 0.5 ml per kg body weight of 20 per cent CCl_4 the total B_{12} level of rabbit serum reached a maximum value within 2–4 days and returned to the pre-treatment level after continuation of the maximum value for several days. In the present experiment injections of CCl_4 were carried out at intervals of 7–10 days. A few ml of blood was obtained by a heart puncture immediately before and 3–5 days after each CCl_4 injection, and the serum B_{12} was determined. A pause of 3 weeks in which no CCl_4 injections were conducted was made between 4th and 5th injections. The overall period of the experiment lasted for 70 days. Control rabbits receiving no CCl_4 injections similarly underwent a heart puncture and the same amount of blood was withdrawn. Typical results are shown in Figs. 1 and 2. As can be seen in Fig. 1, the

FIG. 1 Serum Vitamin B_{12} Levels of Rabbits Repeatedly Injected with CCl_4 . An injected dose was 0.5 ml of 20 per cent CCl_4 solution in olive oil per kg body weight in each injection.

FIG. 2 Serum Vitamin B_{12} Levels of Rabbits Receiving No CCl_4

total B_{12} levels of the serum in two rabbits (Nos. 1 and 2) receiving repeated injections of CCl_4 showed a peculiar change, whereas the levels of the free form of serum B_{12} did not show any significant variation. The change in serum B_{12} was therefore due to the bound form. Fig. 2 shows that the serum B_{12} levels of control rabbits (Nos. 3 and 4) failed to show any variation. In No. 1 rabbit the total B_{12} level was rapidly elevated each time after the first three injections. In No. 2 rabbit the similar elevation occurred each time after the first two injections. In both rabbits, the elevation of the total B_{12} levels did not occur any longer by further injections of CCl_4 , but they fell continuously until they returned to the pre-treatment levels. After a pause of three weeks only a slight elevation occurred after the first two injections in both rabbits. But after the 3rd (the 7th from the beginning of the experiment) injection no further elevation was observed and the levels 70 days after the beginning of the experiment were not so different from the pre-treatment levels. These changes in the total B_{12} levels of the serum in both rabbits after each CCl_4 injection are summarized in Table I. The extent of the elevation in total

TABLE I
Percentage of Serum Vitamin B_{12} Level after Each CCl_4 Injection to That before Injection

Rabbit number	No. of injections						
	1	2	3	4	5	6	7
1	195	166	133	96	115	140	105
2	213	232	60	84	140	159	101

B_{12} levels was expressed as a percentage of the level 3—5 days after each CCl_4 injection to the level immediately before injection. The peculiar changes in the serum B_{12} described above will be clearly shown in the Table.

2. The B_{12} Level of the Liver of Rabbits Repeatedly Injected with CCl_4

To determine the B_{12} level of the liver, all rabbits were sacrificed by ex-

TABLE II
Vitamin B_{12} Content of the Livers of the Rabbits Repeatedly Injected with CCl_4

Rabbit number	B_{12} content of liver	Weight of liver	B_{12} content of whole liver
	$\mu\text{g/g wet tissue}$	g	μg
1	0.16	69	11.0
2	0.18	65	11.7
3	0.55	74	40.7
4	0.49	83	40.7

No. 1 and 2 rabbits were repeatedly injected with CCl_4 as described in the text.
No. 3 and 4 rabbits were not injected.

sanguination 70 days after the beginning of the experiment and the B_{12} level and weight of the livers were estimated. The results are shown in Table II. It will be seen that the weight of the livers of the treated rabbits was markedly diminished and their B_{12} levels per g of wet tissues were very low. The B_{12} content of the whole livers of the treated rabbits was almost a quarter of the values of the control animals. The decrease of the B_{12} content after repeated CCl_4 injections was much more marked than after a single injection which has been described in the previous reports (1, 2).

3. *Pathological Changes of the Liver Tissues of Rabbits Repeatedly Injected with CCl_4*

The livers of the rabbits repeatedly injected with CCl_4 and sacrificed 70 days after the beginning of the experiment were reduced in size and weight, the surface of the livers being markedly roughened by irregular nodules and the consistency being very firm. Microscopic changes in section stained with hematoxylin and eosin were as follows. Interlobular connective tissues were young, loose and markedly hyperplastic, and rich in fibroblasts and blood capillaries. The hepatic cells were markedly irregular both in size and in arrangement, and were divided by the connective tissues into irregular lobules of variable size. The hepatic cells showed a variable degree of necrosis, mixed with newly regenerated hepatic cells. Glycogen was markedly reduced and faintly stained in a few cells in the central region of the lobules. Fat deposition was not so marked as in the case of a single injection of CCl_4 , but a slight scattered deposition was observed among proliferating connective tissues. Ribonucleic acid was stained only in small numbers of hepatic cells comprising newly regenerated cells.

DISCUSSION

In the present report, the changes in the B_{12} content in the serum and liver of the rabbits repeatedly injected with CCl_4 were investigated. The serum B_{12} levels showed a peculiar variation, *i.e.*, the elevation of the serum B_{12} levels occurred each time after the first two or three injections with the intervals of 7–10 days, but it did not occur after further injections, continuing to decline to the pre-treatment levels. After a pause of three weeks, the elevation was again observed each time after two injections, but it was only slight, and no longer observable after the 3rd injection. In the previous papers (1, 2) dealt with the effect of a single injection of CCl_4 on the B_{12} content in the serum and liver, it has been reported that with the degeneration of the hepatic cells into a state of necrosis some of the B_{12} stored in the cells are rapidly released into a blood stream, leading to pathologically high levels of the serum B_{12} . According to Yamamoto *et al.* (7) the elevation of the serum B_{12} levels after CCl_4 administration failed to be observed in rats, whose liver B_{12} levels had been low because of the maintenance of low B_{12} diets, whereas in the rats fed a sufficient diet the elevation occurred. Similar fact was reported by Kato *et al.* (8, 9) that the decrease of the liver B_{12} levels of the patients with cirrhosis of the liver and those with Banti's syndrome following

the portacaval anastomosis was slight in cases whose pre-operation B₁₂ levels were low, while in cases whose levels were high, the decrease was marked. Thus the findings obtained in the present experiment may be explained as follows. Because of the deficiency of the B₁₂ storage of the liver which resulted from the release of the vitamin into blood stream after a first few injections of CCl₄, no release of the vitamin was provoked by further CCl₄ injections, and after a pause, during which the liver B₁₂ storage was slightly recovered, the slight release of the vitamin by the injection occurred. The variation of the serum B₁₂ levels was due to the change of the bound form of the vitamin, whereas the levels of the free form were scarcely changed. In patients with cirrhosis of the liver, the persistent high levels of serum B₁₂ were observed, mainly due to the increase of the bound form. In the present experiment, rabbits repeatedly injected with CCl₄ failed to show persistent high levels of serum B₁₂. Even 70 days after the beginning of the experiment the serum B₁₂ levels remained still within a normal range.

The liver tissues of the rabbits repeatedly injected with CCl₄ lost the normal architectural arrangement of the lobules by active proliferation of fibrous tissues and a part of hepatic cells was necrotic, whereas the rest was regenerated. The B₁₂ content of the liver of these rabbits was markedly lowered. This is in accordance with the fact that the B₁₂ content of the liver is markedly low in patients with cirrhosis of the liver (9, 10).

SUMMARY

The changes in the B₁₂ levels of the serum and liver of the rabbits having received repeated injections of CCl₄ were determined. Each time after the first two or three injections with the intervals of 7–10 days the elevation of the serum B₁₂ levels were observed, but after further injections the elevation did not occur, continuing to decline to pre-treatment levels. After a pause of three weeks the elevation occurred again after the first two injections, but it was only slight, no further elevation being observed by further CCl₄ injections. Even 70 days after the beginning of the experiment no persistent high level of the serum B₁₂ was found. The B₁₂ content of the liver of the rabbits repeatedly injected with CCl₄ fell to almost a quarter of that of untreated animals.

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