Abstract—The therapeutic effect of diisopropyl 1,3-dithiol-2-yldienemalonate (NKK-105) on the fatty liver induced by carbon tetrachloride (CCl₄) was studied. The recovery from elevated liver triglyceride levels induced by CCl₄ required over 20 days in 35 week-old rats, but 14 days in 6 week-old rats. This indicates that 35 week-old rats are useful for studying the therapeutic effect of NKK-105 on fatty liver. In rats with CCl₄-induced fatty liver, NKK-105 lowered the hepatic triglyceride level, accelerated the rate of triglyceride release from the liver, enhanced the incorporation of ¹⁴C-leucine into microsomal protein, and increased the RNA content in microsomes. These data suggest that NKK-105 exerts a curative effect on CCl₄-induced fatty liver by improving the impaired protein synthesis and by promoting lipoprotein secretion.

Diisopropyl 1,3-dithiol-2-yldienemalonate (NKK-105) was synthesized in 1973 by Taninaka et al., at the Chemical Research Center, Nihon Nohyaku Co. Ltd. The pharmacological effects of the compound have been studied in several species of mammals. In the course of the study, NKK-105 was found to be effective against experimental liver injuries. Nakayama et al. showed that NKK-105 increases the flow rate of hepatic blood and bile (1). Liver injuries caused by carbon tetrachloride (CCl₄) and other hepatotoxins were prevented by the administration of NKK-105 (Nokata et al., in preparation). These experimental data suggest that NKK-105 enhances the liver functions.

Studies on the experimental fatty liver induced by the compounds such as CCl₄, ethionine, orotic acid and ethyl alcohol have been of interest from the viewpoint of alleviating fatty liver in humans (2–7). CCl₄-induced fatty liver has been studied extensively to elucidate that the pathogenesis may be eliminated by enhancing the liver functions.

We investigated whether or not NKK-105 would be useful for the elimination of the pathogenesis of CCl₄-induced fatty liver.

MATERIALS AND METHODS

Chemicals: NKK-105 was synthesized and purified to a purity of more than 99.5% with repeated crystallization, at the Chemical Research Center, Nihon Nohyaku Co. Ltd. Radioactive L-[U-¹⁴C]leucine (287 mci/mmol) was purchased from the Radiochemical Center, Amersham (England). Other chemicals were of the reagent grade.

Treatment of animals: Male Sprague-Dawley rats (6 and 35 week-old) were fed laboratory chow (CE-2, Nihon Clea Co.) and tap water ad libitum. CCl₄ (1 ml/kg) was
given s.c. in the form of 20% (v/v) solution in olive oil for 4 days. An equivalent amount of olive oil was given to control rats. NKK-105 was suspended in a 5% arabic gum solution and was given p.o. in a dose of 100 mg/kg daily. The administration of NKK-105 was initiated 72 hr after the final injection of CCl₄. An equivalent amount of 5% arabic gum solution was given to the nontreated NKK-105 rats. The animals were sacrificed by cervical dislocation 24 hr after the last injection of NKK-105. Blood from the cervical vein was collected into a tube and centrifuged at 2,000 r.p.m. for 15 min. The liver was rapidly excised, weighed and homogenized.

Preparation of microsomal protein and RNA fractions: Hepatic microsomes were isolated in the manner described by Ch'ih et al. (8), and were suspended into ice-cold 0.25 M sucrose-10 mM Tris-HCl buffer (pH 7.4) so as to contain about 20 mg of protein/ml. The suspended microsomes were separated by the method of Schmidt and Thanhauser to obtain the protein and RNA fractions (9). The protein fraction was dissolved in 0.1 N NaOH to determine the protein content and the incorporation of amino acids.

Determination of triglyceride release from the liver: The rate of triglyceride release from liver to serum was determined after blocking the removal of triglyceride from serum with Triton WR-1339, according to the procedure described by Abrams and Cooper (10). Triton WR-1339 (1,000 mg/kg) was administered i.v. as a 20% (w/v) solution in saline.

Incorporation of ¹⁴C-leucine in vivo: ¹⁴C-Leucine (50 μCi/kg) was given i.p. 20 min before sacrifice. We then measured the radioactivity incorporated into the microsomal protein, prepared as mentioned above.

Analytical methods: Triglyceride was determined by the method of Van Handel and Zilversmit as modified by Butler et al. (11, 12). Protein was measured by the method of Lowry et al., using bovine serum albumin as standard (13). RNA was determined by orcinol reaction (Schneider) and yeast RNA was used as standard (14).

Determination of radioactivity: The microsomal protein fractions dissolved in 0.1 N NaOH were neutralized with 1 N HCl, and were mixed with Bray's scintillation fluid. The radioactivity was determined using an Aloka LSC-602 type scintillation spectrometer equipped with an external standard. The liquid scintillation counting data were corrected for background (15 to 30 c.p.m.) and counting efficiency (70 to 80%).

Histopathological examination: The left and central lobes of the liver were fixed in 10% formalin and embedded in paraffin. Tissue sections were prepared and stained with hematoxylin-eosin. Frozen sections of the liver fixed in 10% formalin were stained with oil red 0 for lipid.

Statistics: The significance of the difference between two mean values was assessed by Student's t-test.

RESULTS

Recovery from fatty liver induced by CCl₄ in 6 and 35 week-old rats: The administration of CCl₄ increases lipid content in the liver (15). Most of increased lipid is triglyceride (16, 17), thus we measured hepatic triglyceride content as an indicator for fatty liver.

Figure 1 presents the time-course changes in hepatic triglyceride content in 6 and 35 week-old rats after injection of CCl₄. Hepatic triglyceride content in 6 week-old rats reached a peak level one day after the final injection of CCl₄ or on day 1 of observation and then recovered to a normal level within 14 days. In 35 week-old rats, hepatic triglyceride content became maximal on days 3 to 7, decreased gradually thereafter, and on day
20 was about 4 times higher than the normal. In 35 week-old rats, the increased hepatic triglyceride content was maintained for longer periods compared with 6 week-old rats. Thus, 35 week-old rats were used in the study of the therapeutic effect of NKK-105 on fatty liver.

Effect of NKK-105 on triglyceride content in the liver: Figure 2 demonstrates the time-course changes in the hepatic triglyceride content after final injection of CCl4 in both NKK-105-treated and untreated groups. Hepatic triglyceride contents were 13 to 16 mg/g of liver in control rats and did not seem to be affected by the treatment of NKK-105 by itself (10 to 12 mg/g of liver). In CCl4-induced fatty liver, however, hepatic triglyceride levels in the NKK-105-treated group were lower than those in the untreated group at any time of observation. The half life of the elevated hepatic triglyceride level was 3 and 13 days in the treated and untreated group, respectively. In histopathological examination of the liver, NKK-105 reduced the extent of CCl4-induced fatty degeneration.

NKK-105 by itself did not alter the normal architectural pattern of the liver (Figs. 3-5). These results suggest that the increased hepatic triglyceride content by CCl4 falls promptly after initiation of the treatment with NKK-105.

Effect of NKK-105 on the hepatic triglyceride release: Figure 6 shows the effect of NKK-105 on the rate of triglyceride release from liver to serum in CCl4-induced fatty liver. The injection of CCl4 lowered the rate of triglyceride release from the liver to about 1/3 of the normal rate. The rate of release reduced by CCl4 was recovered promptly by the administration of NKK-105. The rate of release in the NKK-105-treated group was normalized within one day, whereas that in the untreated group required three days to recover. The serum level of triglyceride fell following injection of CCl4 and these levels increased with some time lag after the flow rate of triglyceride from the liver recovered (Fig. 7). These results indicate that the rate
Fig. 3. Liver section from a rat treated with NKK-105 for 9 days. The appearance is essentially normal (oil red O, ×54).

Fig. 4. Liver section from a rat 12 days after final injection of CCl₄. Fatty degeneration is evident in the central and periportal zones (oil red O, ×54).

Fig. 5. Liver section from a rat treated with NKK-105 for 9 days from 72 hr after final injection of CCl₄. The area of fatty degeneration induced by CCl₄ is decreased (oil red O, ×54).

Fig. 6. Effect of NKK-105 on the rate of triglyceride release from liver to serum in the rat with fatty liver. Symbols represent: (△) CCl₄ group; (▲) CCl₄+NKK-105 group. Each point represents a mean value of 5 rats. Vertical bars represent S.E. Dashed line shows the normal level (0.14 ± 0.01, Mean ± S.E.). Arrows show CCl₄ injection. Significant difference from control group: *(p < 0.05). For further details see Materials and Methods.

Fig. 7. Effect of NKK-105 on the triglyceride concentration of serum in the rat with fatty liver. Symbols represent: (△) CCl₄ group; (▲) CCl₄+NKK-105 group. Each point represents a mean value of 5 rats. Vertical bars represent S.E. Dashed line shows the normal level (60.2 ± 1.8, Mean ± S.E.). Arrows show CCl₄ injection. Significant difference from control group: **(p < 0.01). For further details see Materials and Methods.

of triglyceride release in CCl₄-induced fatty liver is accelerated by the administration of NKK-105.

Effect of NKK-105 on the RNA content and the incorporation of ¹⁴C-leucine into protein in microsomes: Robinson and Seakins (18) reported that the fatty liver induced by
CCl₄ is related to an impairment of protein synthesis and a consequent decrease in lipoprotein formation. In this connection, we studied the influence of NKK-105 on protein synthesis. As shown in Table 1, the RNA content and the incorporation of ¹⁴C-leucine were decreased by the injection of CCl₄ and were normalized by the administration of NKK-105. Although NKK-105 did not seem to act on the normal rat liver, these data suggest that the drug improves the microsomal protein synthesis disordered by CCl₄.

**DISCUSSION**

As seen in Fig. 1, CCl₄-induced fatty liver was maintained for about 20 days in 35 week-old rats. This indicates that the 35 week-old rat is a suitable experimental model for the study of the therapeutic effectiveness of the compound against fatty liver.

In 35 week-old rats given a subcutaneous injection of CCl₄ daily for 4 days, the hepatic triglyceride content was increased about 12-fold (Fig. 2). In order to avoid the interaction between NKK-105 and CCl₄ in the liver, the administration of NKK-105 was initiated 72 hr after the final injection of CCl₄. Consequently, NKK-105 was shown to be effective for the normalization of the increased triglyceride content in rats with fatty liver.

The decreased secretion of lipoprotein from the liver was reported to be associated with the fatty liver induced by CCl₄ (19). In the present study we ascertained the effect of NKK-105 on the rate of triglyceride release from the liver. This rate of release was approx. 0.14 mg of triglyceride/min/100 g body weight in normal rats. This value was comparable to that reported by Abrams and Cooper (10). The rate was significantly lower in the group with CCl₄-induced fatty liver than in the control, which suggests a decrease in the release of lipoprotein from the liver to the serum. Promptly after the treatment with NKK-105, the rate of triglyceride release from liver to serum was restored from the decreased levels (Fig. 6). It follows that NKK-105 accelerates the flow rate of lipoprotein from the liver to the serum. The following causative factors are thought to retard the triglyceride release from liver to serum: 1) decreased synthesis of apoprotein which is a constituent of lipoprotein, 2) decreased assembly of very-low-density lipoprotein and 3) decreased release of very-low-density lipoprotein. It is also known that degradation of liver polysomes, decrease in RNA and impairment of protein synthesis are induced by the administration of CCl₄ (20–24). The slowdown of apoprotein synthesis, therefore, is considered to be a primary factor. For this reason, we studied the effect of NKK-105 on protein synthesis.

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**Table 1. Effect of NKK-105 on microsomal RNA content and incorporation of ¹⁴C-leucine into microsomal protein**

| Group          | Microsomal RNA (mg/g of liver) | Incorporation of ¹⁴C-leucine (dpm/mg of protein) |
|----------------|--------------------------------|--------------------------------------------------|
| Control        | 6.19±0.28                      | 768±64                                           |
| NKK-105        | 6.43±0.72                      | 789±93                                           |
| CCl₄           | 5.50±0.21                      | 526±43*                                         |
| CCl₄+NKK-105   | 6.58±0.24**                    | 699±28                                           |

Each value is a mean±S.E. of 5 rats. CCl₄ (1 ml/kg) was given s.c. for 4 days. NKK-105 (100 mg/kg) was administered p.o. for 3 days, starting 72 hr after the final injection of CCl₄. Significant difference from control group: *(p<0.05). Significant difference from CCl₄ group: ***(p<0.01). For further details see Materials and Methods.
synthesis in microsomes.

As shown in Table 1, the microsomal RNA content and the incorporation of 14C-leucine into microsomal protein were significantly decreased in CCl₄-induced fatty liver in comparison with findings in the normal liver. These values reverted to normal following the administration of NKK-105. Thus, the protein synthesis in microsomes impaired by CCl₄ was repaired by NKK-105.

In conclusion, the action of NKK-105 against fatty liver induced by CCl₄ may be summarized as follows: 1) partial repair of the lowered protein synthesis in microsomes, 2) acceleration of the rate of triglyceride release from liver to serum and 3) decrease of the triglyceride content in the liver.

Further studies are in progress with a view to more accurately characterizing the mechanisms of action of NKK-105 in healing CCl₄-induced fatty liver.

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