INTRODUCTION

Various rodent models have been developed to study mechanisms of organ damage after chronic alcohol intake (for recent reviews, see de la Hall et al., 2001; Järveläinen and Lindros, 2002). To optimize the daily ethanol intake, Lieber and co-workers developed the ‘Lieber–DeCarli’ liquid diet protocol, but blood ethanol levels and organ lesions were moderate (Lieber and DeCarli, 1982). Higher blood ethanol levels were achieved with the intragastric ‘forced-feeding’ liquid diet model (Tsukamoto et al., 1985). This in combination with a diet rich in polysaturated fatty acids and low in carbohydrates resulted in marked liver lesions (Nanji et al., 1989). However, the intragastric delivery model is expensive and labor-intensive. We developed an oral administration model of a similar liquid diet to rats (Lindros and Järveläinen, 1998) and were able to reproduce most of the hepatic changes observed in rats receiving the diet intragastrically (Ronis et al., 2004).

The purpose of the present study was to develop a method for efficient chronic ethanol administration to mice, in order to study specific pathogenetic factors in genetically modified rodent strains. We originally attempted to apply our oral liquid diet rat administration model, but we encountered technical problems. Lack of convenient equipment for down-scaled liquid diet administration made us consider alternative approaches. Previously, rats had been offered ethanol solution in agar gel blocks and in addition normal solid food (Landrigan et al., 1989; Gentry-Nielsen et al., 2001). We reasoned that ethanol-containing liquid diet could be made semi-solid by addition of agar and that this gel could be offered without the need of specially designed liquid diet bottles.

MATERIALS AND METHODS

Animals

Male C57/Bi mice, initially weighing 24–27 g, were obtained from Harlan, the Netherlands. They were individually housed in M2 plastic cages and fed standard rodent food ad libitum for 5 days until initiation of the gel diet experiment. Mice received agar gel diet with (n = 12) and without (n = 8) ethanol for 6 weeks. The weight of the mice was recorded daily. These mice served as controls in a larger study including C57/Bi-derived deficient KO mice. These mice behaved similarly as the wild-type controls with respect to consumption of diet, weight gain and blood ethanol levels (data not shown).

Preparation and delivery of agar gel diet

We prepared a modified high-fat/low-carbohydrate liquid diet where the commercial Lieber–DeCarli (LD 101A; Purina Mills, Richmond, IN) provides 50% of the calories as previously described (Lindros and Järveläinen, 1998). Briefly, the fat content is increased from 35% (calories) to 44% by adding extra corn oil and the protein content maintained at 16% by adding casein (technical grade; Sigma, St Louis, MO). Vitamins and minerals are also added to equal the composition of the Lieber–DeCarli diet, but no carbohydrate, so that its content is reduced from 11 to 5.5%. Ethanol provides 34.5% of the calories. The control diet contains 40% carbohydrate (maltodextrine) to equecalorically replace ethanol. The exact composition of the LD101 diet, including its content of vitamins and minerals, can be found at the address http://www.dyets.com/710260.htm. Agar [Lab M Agar No 2, Amershams MC 6, 0.5% (w/w)] was added as described below.

Gel diet was prepared twice weekly as follows: agar powder was suspended and mixed in half of the final water volume and stored at +4°C. Loss of ethanol during the preparation and storage was found to be negligible.

A portion of the ethanol diet (20–25 g) was inserted into Falcon tubes equipped with an ~2 × 2 cm opening (Fig. 1). The Falcon tube was mounted in a tilted position inside the pellet grid of the cage using metal strings. The daily diet consumption was determined by weighing the Falcon tube.
Control mice also received their diet in Falcon tubes. All mice also had access to a water bottle during the experiment.

Ethanol gel diet intake was recorded daily and control mice pair-fed an equicaloric amount of the control diet the subsequent day. During the first 2 weeks the concentration of ethanol in the diet was gradually increased from 2 to 5.3% (final). Pilot experiments revealed that the window for an optimal ethanol concentration in the diet is narrow in mice. A diet with 4.5% ethanol produced low blood ethanol levels while 6% caused reduced diet intake and gradual loss of weight.

**Biochemical assays and liver histopathology**

Blood ethanol levels were determined by sampling from vena saphena (25 µl) from groups of mice at weekly intervals. After 6 weeks of treatment, mice were anaesthetized with sodium pentobarbital (60 mg/kg i.p.), blood samples collected by heart puncture and plasma separated and stored at −20°C. Pieces of liver were collected in buffered formalin, embedded in paraffin, cut in 6 µm sections and stained with hematoxylin/eosin. Steatosis was graded blindly from 0–4 as follows: 1 = <25% of cells containing fat, 2 = 26–50%, 3 = 51–75%, 4 = >75%.

The concentration of ethanol in blood and gel diet was determined by head-space gas chromatography (Hu et al., 1995). For assay of liver triglycerides as glycerol, 1 ml of liver methanol–chloroform mixed homogenate was washed with sodium chloride, the resultant extract was dried and dissolved in 200 µl of tetraethylammoniumhydroxide (1:28 with 95% ethanol), incubated at 60°C for 30 min and mixed with 200 µl 50 mM HCl. Glycerol and serum alanine aminotransferase (ALT) activity were measured enzymatically by using commercial kits (Boehringer-Mannheim, Germany).

The data are expressed as means ± SD. Student’s t-test was used to test statistical difference between groups. Pathological scores were compared using the Mann–Whitney test. A P-value < 0.05 was considered statistically significant.

The study had been approved by the Committee for Animal Experimentation of the National Public Health Institute in Helsinki, Finland.

**RESULTS**

**Reducing ethanol evaporation by using Falcon tubes**

Based on a previous publication (Gentry-Nielsen, 2001) a pilot study was initially performed with agar gel on a Petri dish. However mice on this regimen had very low blood ethanol levels (results not shown). Major loss by evaporation was considered the most probable reason for this. Assay of ethanol from a 2 × 2 × 1 cm piece of gel left on a Petri dish in the animal house indeed demonstrated that in 6 h almost 50% had evaporated and at 24 h less than 20% was left (Table 1). Attempts were made to compensate for the loss of ethanol by evaporation by increasing the ethanol content in the diet to up to 10%. However, the blood ethanol levels remained low. Consequently, the Falcon tube delivery system was developed as a means of reducing evaporation.

To determine the loss of ethanol from a normal portion of ethanol diet in Falcon tubes, the intake of gel diet by the mouse was mimicked. Fresh gel diet was provided in the Falcon tube to mice. About 1 g of gel was removed as a 2–3 mm thick surface layer (representing the amount of gel diet the mouse would eat) at 6, 17 and 24 h. At 6 h, 25% of ethanol was lost. However, at 17 and 24 h there was no additional loss (26%). At first glance this was surprising. However, since in the mean time the mouse had been eating deeper into the gel piece, fresh gel had been exposed. Thus, although exact mimicking of the eating behavior of the mouse is impossible, our data indicate that loss by evaporation from the surface is limited and that enough ethanol is consumed to result in consistently elevated blood ethanol levels. Although the design of the Petri dish experiments was slightly different, and thus the results not exactly comparable with the Falcon tube data, the data nevertheless show that much more ethanol evaporates from gels on Petri dishes.

**Blood ethanol levels and liver changes**

Both groups of mice gained weight during the 6 week gel diet regimen, but the control mice more (6.7 ± 1.9 g) than the

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<th>0 h</th>
<th>6 h</th>
<th>17 h</th>
<th>24 h</th>
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</thead>
<tbody>
<tr>
<td>Petri dish</td>
<td>55.9 (100%)</td>
<td>30.7 ± 6.5 (55%)</td>
<td>–</td>
<td>9.3 ± 2.0 (17%)</td>
</tr>
<tr>
<td>Falcon tube</td>
<td>55.9 (100%)</td>
<td>42.2 ± 3.5 (75%)</td>
<td>41.6 ± 1.8 (74%)</td>
<td>41.2 ± 3.4 (74%)</td>
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The ethanol concentration is given in g/l gel (n = 3–6).
ethanol-treated mice (0.8 ± 1.8 g) (Fig. 2). The average daily diet intake by ethanol-treated mice during the 4 week period with the final concentration of ethanol in the diet (5.3%) was 16.3 ± 2.2 g. There were minor day-to-day fluctuations but the average weekly intake was quite stable. The controls were always given the equivalent amount of control diet. Occasionally some control animals did not eat all diet offered. The daily intake of ethanol diet corresponded to 23–24 g absolute ethanol per kg body weight. The additional average intake of fluid from the water bottle was low both for ethanol-treated (1.6 ml) and control mice (1.4 ml).

Blood samples for ethanol determination were taken between 8 and 9 am on four different occasions at weekly intervals. The average blood ethanol levels (means of 4–10 samples) calculated from sampling at week 2, 3, 4 and 5, were 14.1 ± 13.1, 43.9 ± 14.8, 76.9 ± 28.8 and 27.4 ± 18.8 mM, respectively. Note that different animals served as blood donors at different time points.

Histopathological evaluation of liver samples taken at termination revealed that after 6 weeks of ethanol gel consumption, the mice had developed marked mixed micro- and macrovesicular steatosis, reflected in an increase in the liver/body weight ratio and triglycerides (Table 2). Occasional mononuclear infiltration was also seen (results not shown). In addition, the serum activity of alanine aminotransferase was more than doubled.

**DISCUSSION**

This study describes a new simple method for chronic ethanol administration to rodents. A nutritionally adequate liquid diet containing 5.3% ethanol is made into a gel by addition of a small amount of agar. To reduce evaporation of ethanol the gel is offered in plastic tubes equipped with an eating opening. The amount of gel diet consumed by the mice is high enough to result in sustained high blood ethanol levels, which in 6 weeks results in significant liver steatosis and elevated plasma ALT activities.

Although agar blocks have been used before as a vehicle for ethanol administration, the combination of liquid diet and ethanol is new. There are several advantages with this model. The method is simple and does not require any special equipment. The diet does not need to be prepared daily. Compared to ordinary liquid diet provided in bottles, there is no loss of diet due to bottle leakage or layering of diet during storage, which can be a problem with liquid diet. In the present study, control animals also received their daily portion in Falcon tubes, but the procedure could have been further simplified by simply offering the gel on Petri dishes. Furthermore, the agar gel contains enough water to satisfy the animal’s daily fluid requirement, as evidenced from the minimal extra intake from the water bottle.

Evaporation of ethanol from the gel proved to be a greater problem than we had anticipated on the basis of published studies. Neither the study by Landrigan et al. (1989) nor the later study by Gentry-Nielsen et al. (2001) appreciated the magnitude of this problem. Anticipating little evaporation, these studies reported values of daily intake of ethanol exceeding 40 g/kg body wt. These values are clearly too high, since the rat cannot metabolize more than 14–18 g/kg body wt in 24 h (Lindros et al., 1983). Thus, evaporation of ethanol from the agar blocks must have been massive, and consumption of ethanol from agar blocks cannot be calculated without correction for this.

In spite of pair-feeding, the control mice gained more weight in comparison with those given ethanol diet. This ‘energy wastage syndrome’ has long since been recognized (Pirola and Lieber, 1976), but the phenomenon is still not fully understood (Suter, 2000). The phenomenon may be accentuated in mice due to their fast metabolism. There also were much larger individual variations in blood ethanol levels during the experiment as compared to rats on an ordinary liquid diet. We suggest that this also can be related to the much faster rate of ethanol metabolism in the mice, so that occasionally most of the ethanol has disappeared before the animal eats more diet.

Finally, we suggest that although the present study was based on mice, it should be applicable for other rodents.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Liver/body wt ratio (%)</th>
<th>Steatosis score (0–4)</th>
<th>Triglycerides (mg/g)</th>
<th>Serum ALT (U)</th>
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<tbody>
<tr>
<td>Control (n = 8)</td>
<td>3.8 ± 0.2</td>
<td>1.6 ± 0.5</td>
<td>2.40 ± 0.35</td>
<td>7.9 ± 3.3</td>
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<tr>
<td>Ethanol-treated (n = 12)</td>
<td>5.1 ± 0.4*</td>
<td>2.4 ± 0.5*</td>
<td>4.48 ± 1.45*</td>
<td>16.6 ± 5.2*</td>
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*P < 0.05 compared to controls.
as well. For instance, by delivering the agar gel in bigger plastic tubes (200 ml) equipped with suitably sized eating holes the model should be useful in chronic studies with rats as well.

REFERENCES


