Rapid Publications

Normal Testicular Structure and Reproductive Function in Deermice Lacking Retinol and Alcohol Dehydrogenase Activity

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Abstract. It was found that a strain of deermice (*Peromyscus maniculatus*), which genetically lacks liver alcohol dehydrogenase activity also displays no such activity in the testis and is devoid of the enzyme activity that converts retinol to retinal, both in liver and in the testis; nevertheless, these animals exhibit normal reproduction and testicular histology. Therefore, one must reconsider the theory that the testicular atrophy and aspermatogenesis commonly found in alcoholics is due, at least in part, to interaction of ethanol with these enzyme activities in the testis.

Introduction

Testicular atrophy is frequently observed in alcoholics (1) and in experimental models of alcohol abuse (2). Originally, the testicular lesion was attributed to associated liver disease but since it was also observed in alcoholics with relatively mild liver injury (3) it has been postulated that the lesion can be attributed, at least in part, to a toxic effect of alcohol itself. Although retinoic acid can maintain general effects of the vitamin (such as growth), a specific form of vitamin A, retinol (but not retinoic acid) is essential for spermatogenesis and normal testicular function (4). Since the conversion of retinol to retinal in testicular homogenates is inhibited by ethanol (5), it was postulated that the biochemical mechanism for the sterility of alcoholics is this inhibitory effect. It has also been proposed that ethanol may affect steroidogenesis in the testis by an alcohol dehydrogenase (ADH)[1]-mediated NAD/NADH shift (1, 6). Verification of these hypotheses became possible when a strain of deermice (*Peromyscus maniculatus*) was discovered, which genetically lacks ADH (7). In this study, we confirmed that these animals lack ADH in their liver; we demonstrated that they also are devoid of this enzyme in the testes and that they lack the enzyme activity that converts retinol to retinal in both liver and testes. Yet these animals have normal reproductive functions and testicular histology.

Methods

Animals. Deermice (*Peromyscus maniculatus*) used in the present study had the following genotype for the liver ADH: ADH^F/ADH^F (ADH-positive) and ADH^N/ADH^N (ADH-negative). The ADH-positive strain had a normal liver ADH activity while the ADH-negative strain had no detectable ADH activity on zymograms or in spectrophotometric assays and, moreover, exhibited no antigenically cross-reacting material in immunochemical tests using monospecific anti-ADH antisera (7). These animals were bred at the Alcohol Research and Treatment Center of the Bronx Veterans Administration Medical Center and were fed laboratory mouse chow (Ralston Purina Co., St. Louis, MO).

The animals were killed by cervical dislocation and livers and testes were quickly excised. Liver homogenates were prepared with 1.15% KCl solution. The following procedures were carried out at 0°–4°C: the liver homogenate (20%) was centrifuged at 10,000 *g* for 20 min. The supernatant was centrifuged at 105,000 *g* for 1 h. The resulting supernatant (cytosol) was used as source for enzyme assays. Cytosol was also prepared from pooled testes (at least 12 testes in each pool) following the above procedure except that the homogenate was 50%. Cytosolic ADH activity was determined by the method of Bonnichsen and Brink (8), modified as follows: final ethanol concentration was 50 mM and 1 mg of NAD was used per milliliter. Retinol dehydrogenase (RDH) activity was determined according to Mezey and Holt (9).

The enzyme activities were also determined by isoelectric focusing on polyacrylamide gels using LKB Ampholine PAG plates (LKB In-

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1. *Abbreviations used in this paper:* ADH, alcohol dehydrogenase; RDH, retinol dehydrogenase.

struments, Inc., Stockholm, Sweden) containing 2.4% ampholytes, pH 3.5–9.5, on an LKB Multiphor flatplate system at 4°C. The anode buffer was 1 M H_3PO_4 and the cathode buffer was 1 M NaOH. Samples of pooled cytosol (100 μl for the liver and 200 μl for the testis) were applied to the gel by absorbing the sample onto 5 × 10-mm pieces of filter paper. Plates were run at a constant power of 20 W (power supply model 1420B, Bio-Rad Laboratories, Richmond, CA) for 45 min, after which the sample application papers were removed. Focusing was then continued for 45 more min. At the end of the run, a 1-cm wide strip was cut from the short end of the plate and divided into 1-cm long segments. Each segment was placed in 2 ml distilled water and the pH was read after 1 h. This permitted determination of the pH gradient of the plate. The gel was then stained with a modification of the procedure of Fine and Costello (10): the staining solution consisted of 75 ml of sodium phosphate buffer (0.2 M, pH 7.4 at 37°C), NAD 50 mg, phenazine methosulfate 4 mg, and nitroblue tetrazolium 12 mg with either ethanol 110 mM or retinol 0.4 mM as substrates; pH 7.4 was adopted because it was found to be optimal for RDH activity. Two control preparations were used without substrate (with or without 1 mM 4-methylpyrazole). As reference we used purified horse liver ADH (10 mg/ml) (Boehringer-Mannheim Biochemicals, Indianapolis, IN). 1 μg was dissolved in 200 μl water and 10 μl applied to the gel. The gels were incubated at 37°C for 10 min. Protein was determined by the method of Lowry et al. (11).

Testicular tissue was also prepared for light and electron microscopy with conventional techniques in use in our laboratory.

Results

Using the spectrophotometric method, ADH-positive animals had ADH activity in the cytosol varying from 5 to 20 nmol/min per mg protein and 0.05 in the testes. Corresponding RDH activity varied between 1 and 4 nmol/min per mg protein in the liver. The values were at the limit of detection in the testes. In ADH-negative deermice, no detectable activity was found in either liver or testes for both enzymes.

By electrofocusing, as shown in Fig. 1, in ADH-positive animals both liver and testes had clear-cut ADH and RDH activity in the cytosol, focusing at a pH of ~10.6. In the absence of substrates (data not shown) only a faint band appeared, most likely due to traces of ethanol in the reagents (7); it was completely suppressed by 4-methylpyrazole. By contrast, in the ADH-negative animals no activity was detected for both enzymes in either tissue under any conditions.

By light (Fig. 2) and electron microscopy both ADH-positive and ADH-negative animals had normal testicular histology.

Reproductive capacity, determined by the number of offsprings per breeding couple, was comparable in both ADH-positive and ADH-negative animals. No obvious difference was observed in development of sexual organs between ADH-negative and ADH-positive male deermice.

Discussion

This study demonstrates that deermice which lack ADH in their liver also display no such activity in the testes and that this deficiency is associated with the absence of RDH activity in both tissues. Nevertheless, these animals reproduced normally and their testes displayed normal histology.

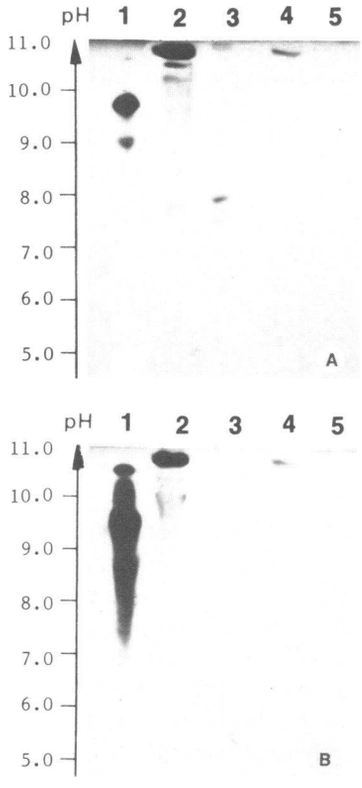

Figure 1. Electrofocusing of cytosolic ADH (*A*) and RDH (*B*) activities in liver and testis of ADH-positive and ADH-negative deermice. ADH-negative animals were found to be lacking these two enzyme activities. Horse liver ADH (lane *1*) (used for reference); liver of ADH-positive (lane *2*) and -negative (lane *3*) animals; testis of ADH-positive (lane *4*) and -negative (lane *5*) deermice.

These observations are of particular relevance to proposed theories for the pathogenesis of testicular atrophy in alcoholics.

As reviewed elsewhere (1) hypogonadism in the alcoholic has been recognized for decades but its mechanism has been the subject of debate. Originally, the hypogonadism and testicular atrophy found in alcoholics was thought to be due to damaged hepatic function, since testicular atrophy could be seen in severe liver disease not due to alcohol abuse. However, a pathogenic role for alcohol has also been postulated and its involvement implicated at various levels. Alterations at the level of the hypophysis and hypothalamus have been emphasized by Cicero et al. (12). A role for malnutrition, particularly deficiency in vitamin A and zinc, have also been highlighted by various studies: since zinc deficiency produces hypogonadism in experimental animals and since vitamin A is necessary for spermatogenesis and normal testicular function, it was naturally postulated that deficiencies in zinc and vitamin A may play a role in the hypogonadism of alcoholics (13).

One leading new hypothesis proposed over the last decade to explain hypogonadism in the alcoholic is the direct testicular toxicity of ethanol (3, 5). Consistent with the latter hypothesis is the observation that alcoholics may develop severe gonadal deficiencies even in the absence of marked liver injury: indeed it was shown by Van Thiel et al. (3) that alcoholics with minimal

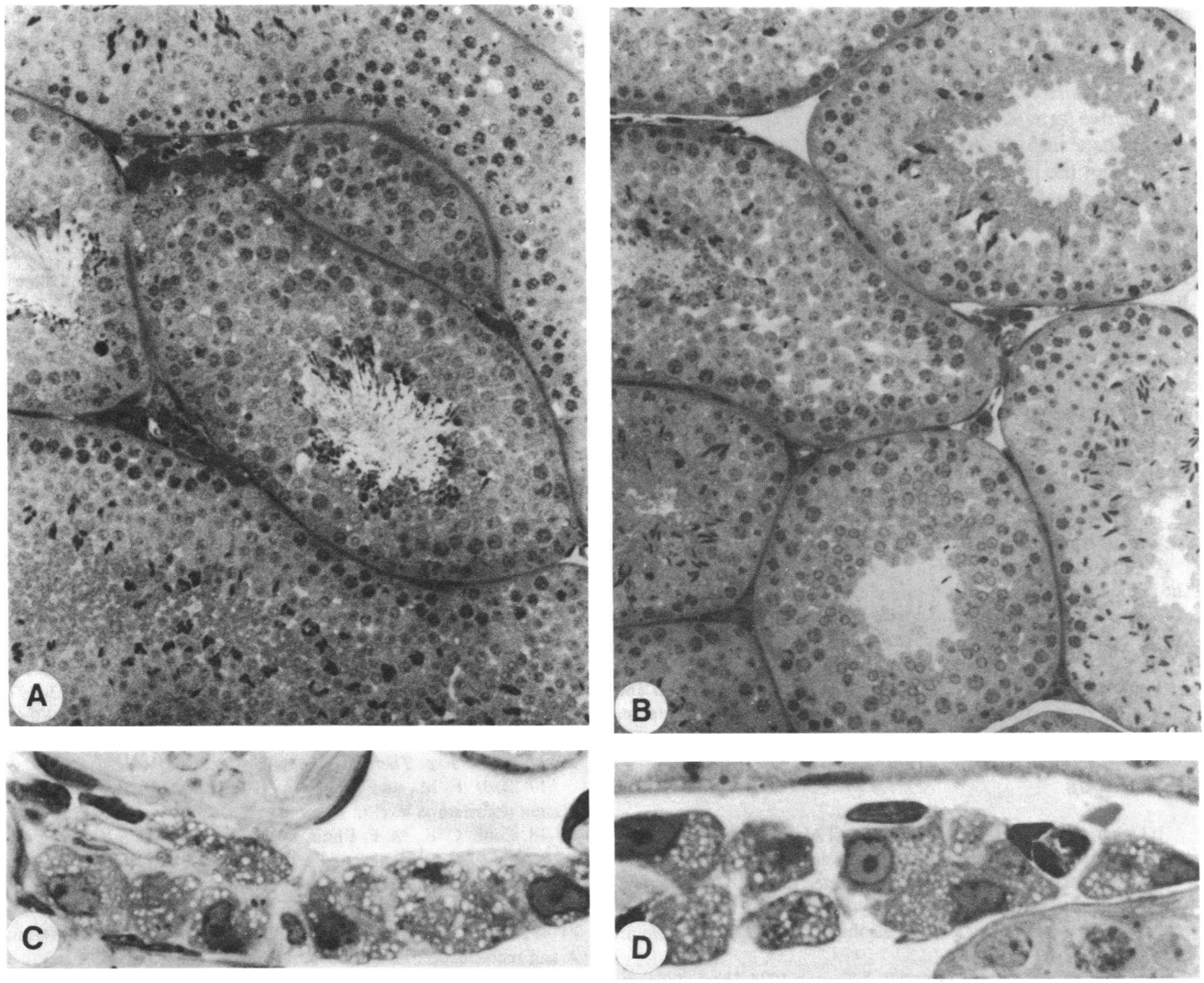

Figure 2. Testes of deermice. The seminiferous tubules of ADH-positive (*A*) and ADH-negative (*B*) animals as well as the Leydig cells of ADH-positive (*C*) and ADH-negative (*D*) deermice are normal. (1-μm thick Epon sections, toluidine blue staining; *A* and *C* × 270; *B* and *D* × 675.)

hepatic lesions could develop clinical and biochemical hypogonadism. It appeared therefore that the commonly accepted direct relation between abnormal sexual changes and cirrhosis was an oversimplification. Experimental work in alcohol-fed rats (2, 5, 14) confirmed clinical impressions. The hypothesis of a direct toxicity of ethanol was also supported by the demonstration that alcohol ingestion suppresses plasma testosterone levels in nonalcoholic and alcoholic volunteers without preexisting liver disease (15, 16) and in animals (14, 17). The direct toxicity of ethanol was also demonstrated by the suppression of testicular functions with alcohol and acetaldehyde in the isolated perfused rat testis (18).

How alcohol, or its metabolite acetaldehyde, causes testicular injury is not clear but several mechanisms have been proposed. One attractive hypothesis has been formulated by Van Thiel et al. (3): they demonstrated that ethanol inhibits testicular conversion of retinol to retinal. From earlier work (19) it was assumed that, though retinoic acid could perform functions of retinol such as effect on growth and maintenance of epithelia, it was without function in the testis and the visual cycle of the eye. Though results of Appling and Chytil (20) now suggest that retinoic acid, either derived directly from the bloodstream or from the oxidation of retinol, acts on the Leydig cells to maintain serum testosterone, it appears that the degeneration of the ger-

minal epithelium, seminiferous tubules, and the cessation of spermatogenesis in vitamin A-deficient rats is not prevented by retinoic acid in vivo (4, 21), although some effects were found in vitro (22). In the eye, it has been well established that retinal is the active form of retinol and therefore, it appeared attractive to postulate that direct effects of ethanol in the testes could result from the interference by ethanol with the conversion of retinol to retinal (3, 5). As an alternative or supplemental mechanism for the toxicity, it has also been proposed that ethanol produces an alteration of steroidogenesis by causing a NAD/NADH shift (1, 6, 14). Both these postulated mechanisms involve the activity, in the testes, of either ADH (to cause a local redox change) or RDH (to convert retinol to retinal). It is apparent that the demonstration, in the present study, of normal reproductive function and testicular structure despite the absence of these two enzyme activities requires serious reconsideration of the proposed hypotheses and that some other mechanism for the direct effects of ethanol on the testes must now be searched for.

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