Complete prevention of arthritis was brought about by a transfer of 5×10^8 cells at 8 weeks in females and 16 weeks in males (table).

The maximum score of arthritis in recipient rats which received 0.3 mg mycobacterial adjuvant immediately after cell transfer. The cells were obtained from popliteal lymph nodes draining the injection site of 0.003 mg of this adjuvant 4–16 weeks before

Sex of donors	Number of cells transfered	Cells 4 weeks	8 weeks	16 weeks
Male	5×10^8		28.0 ± 5.7*(7/7)	0 (0/8)
	5×10^7		39.3 ± 0.8 (5/5)	25.2 ± 5.1 (4/4)
	0		37.1 ± 0.8 (4/4)	36.6 ± 0.5 (4/4)
Female	5×10^8	29.4 ± 5.6 (8/8)	0 (0/8)	
	5×10^7	38.4 ± 1.3 (5/5)	22.8 ± 2.3 (4/4)	
	0	37.8 ± 1.1 (5/5)	36.7 ± 1.0 (5/5)	

* Mean maximum joint score ± SE. ( ) Number of animal with arthritis.

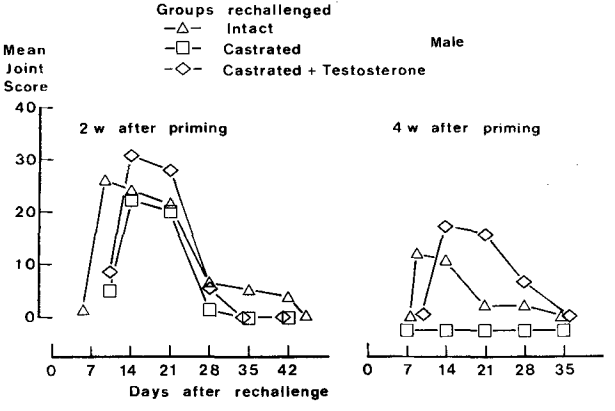

Figure 3. The clinical scores of arthritis in male rats (6–8 in each group) rechallenged with 0.3 mg mycobacterial adjuvant at 2–4 weeks after the primary challenge with 0.003 mg of this adjuvant. Castration was performed 2 weeks before priming. A testosterone pellet was implanted 2 days after castration and removed 7 days after priming.

Discussion. Unresponsiveness to mycobacterial adjuvant is due to an inhibitory response by suppressor cells, presumably T cells[4,5]. The present study indicated that male rats were more resistant than female rats in the induction of unresponsiveness in highly susceptible DA rats. The induction time of unresponsiveness in male rats shifted toward the female type after castration. An application of testosterone to castrated male rats could revert the induction time to the male type. Testosterone removed before priming had no such restorative effect. This suggests that testosterone acts suppressively on the kinetics of the development of suppressor cells. However, the definitive clarification of the mechanisms of testosterone action on suppressor cells must await further study. The transfer study also presented evidence indicating a sex difference in the incubation time for the development of suppressor cells. Although genetic control of the susceptibility to AA has been reported by other investigators[7,8], the present study suggests that testosterone could be a factor modulating the manifestation of this disease. Modulation of the expression of autoimmunity by androgen action on the thymic-dependent regulatory mechanisms has been reported in MRL/lpr and NZB × NZW F_1 mice [9,10].

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Covalent binding of aflatoxin B_1 to liver DNA in rats pretreated with ethanol

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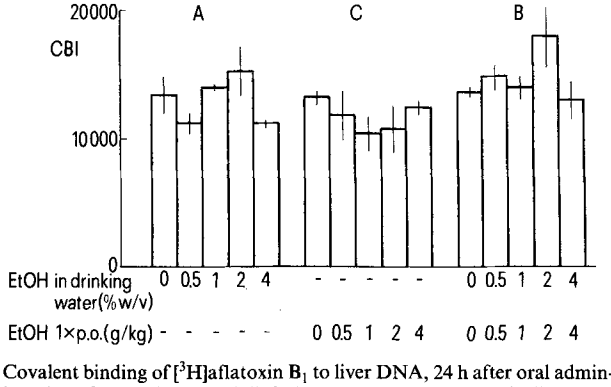
Summary. Male Fischer F-344 rats were given ethanol in the drinking water and/or by single oral administration. Following this, the animals received p.o. 100 ng/kg of the hepatocarcinogen [^{3}H]aflatoxin B_1 (AFB_1). 24 h later, the level of DNA-bound AFB_1 was determined in the liver and was found not to be affected by any type of ethanol pretreatment. A cocarcinogenic effect of ethanol in the liver is therefore unlikely to be due to an effect on the metabolic activation and inactivation processes governing the formation of DNA-binding AFB_1 metabolites.
Key words. Carcinogenesis; DNA; covalent binding; aflatoxin; ethanol.

Although alcohol is listed as a factor contributing to between 2 and 4% of all human cancer deaths[4], pure ethanol has so far yielded negative results in animal tests for carcinogenicity (reported in Obe and Ristow[5]). Alcohol must therefore be considered to be a cofactor in the etiology of cancer[6]. One of the mechanisms postulated to explain its cocarcinogenic activity is the ability of ethanol to interact with drug-metabolizing enzyme systems involved in the activation of procarcinogens to reactive, DNA-binding intermediates. Enzyme activities can either be induced after repeated administration of ethanol or be inhibited by ethanol itself (see reviews by Lieber et al.[7] and McCoy et al.[8]). The susceptibility of the enzymes to this latter effect seems to be organ-specific, as it has recently been found that the first-pass clearance of dimethyl- or diethylnitrosamine[9] and of methylbenzylnitrosamine[10] is inhibited by ethanol in the liver but not in the oesophagus. These results might explain mechanistically some of the results describing a modulation of nitrosamine carcinogenesis by ethanol (Lieber et al.[7] McCoy et al.[11], Pour et al.[12]), including a shift of the target organ[13].

Since one of the target organs for the cocarcinogenic activity of

ethanol in man is the liver[4], and because aflatoxin B_1 (AFB_1) is a potent hepatocarcinogen in the rat and a probable hepatocarcinogen for man[14] we wanted to know whether any synergistic effect of single or repeated ethanol treatment on the covalent binding of AFB_1 to liver DNA could be found. DNA binding has been shown to be an important early step in carcinogenesis by a large number of organic chemicals[15], and there are a number of reports showing that the decrease in liver tumor induction by AFB_1 in rats pretreated with a protein-deficient diet[16] or with phenobarbitone[17,18] was paralleled by a decrease in total binding of AFB_1 to liver DNA. The availability of radiolabeled AFB_1 of very high specific activity and the fact that the DNA-binding activity of AFB_1 to rat liver DNA is among the highest ever determined for a genotoxic carcinogen[19], made it possible to work with a very low AFB_1 dose of 100 ng/kg which is of the order of environmental exposure levels in some human populations[20].

Materials and methods. Male Fischer F-344 rats weighing about 180 g at the beginning of the experiment were obtained from Charles River (Sulzfeld, FRG). Three experiments were performed: In experiments A and B, groups of two rats received ethanol (p.a.: Merck, Darmstadt, FRG) in the drinking water for 10 days, at concentrations of 0.5, 1, 2, and 4%(w/v). The effective daily intake of alcohol in the different dose groups was measured to be 0.44, 0.89, 2.1, and 2.68 g/kg (experiment A) and 0.52, 1.22, 1.91, and 3.4 g/kg (experiment B). The pretreatment did not result in any sign of toxicity, such as abnormal behavior or decreased body weight gain. After 10 days, the animals in experiment A were given p.o. 100 ng/kg b.wt (about 0.5 μCi/rat) [^{3}H]AFB_1 (8 Ci/mmole; Moravek Biochemicals, City of Industry, CA), as an aqueous solution of 50 ng/ml. The animals in experiment B received the same dose of [3H]aflatoxin together with 0.5, 1, 2, or 4 g ethanol per kg b.wt in water. Here, alcohol had been withdrawn from the drinking water for 16 h before the single administration of ethanol which represented about one daily dose of the pretreatment regimen. In experiment C, four groups of 2 rats recieved a single oral dose of [^{3}H]AFB_1 (100 ng/kg) together with 0, 0.5, 1, 2, and 4 g/kg ethanol. In each experiment, two control rats received water instead of alcohol. 24 h after the administration of the carcinogen, the animals were killed by an overdose of ether and the livers were excised. DNA was isolated as described[21] and the specific radioactivity was determined. Acid treatment of the DNA to liberate the purines and reverse-phase HPLC of the hydrolysate[22] revealed that the radioactivity did not co-elute with the optical density of adenine or guanine but appeared without optical density at later positions known to contain the more lipophilic purine-aflatoxin adducts[23]. All DNA radioactivity therefore represented covalently bound aflatoxin and the specific activity of DNA could be expressed in the units of the Covalent Binding Index[15], CBI = (μmole AFB_1 bound per mole DNA nucleotide)/(mmole AFB_1 administered per kg b.wt).



Covalent binding of [^{3}H]aflatoxin B_1 to liver DNA, 24 h after oral administration of 100 ng/kg to male F-344 rats pretreated subchronically and/or acutely with various doses of ethanol (EtOH). The binding level is expressed in CBI units[15], CBI = (μmoles AFB_1 bound per mole DNA nucleotide)/(mmoles AFB_1 administered per kg b.wt). The vertical bars indicate 1 SD derived from two animals.

Results and discussion. The covalent binding of aflatoxin B_1 to liver DNA of the six untreated rats (two in each experiment A, B, and C) resulted in a CBI value of 13,500 ± 700 (1 SD), in good agreement with the values determined earlier[19]. None of the pretreatment regimens with ethanol resulted in a dose-dependent change (fig.). Statistical analysis of the data with Spearman's rank correlation coefficient revealed a $p < 0.1$ with respect to a decrease in DNA binding in experiment C, but only up to a single ethanol dose of 2 g/kg DNA. The CBI obtained after 4 g/kg was almost back to control values. Pretreatment for 10 days (experiment A) revealed a tendency towards an increase, but, again, the highest dose tested gave rise to normal values. It was not surprising then to see that the combination of chronic and acute ethanol treatment (experiment B) balanced out the two trends mentioned above. The higher CBI at 2% ethanol in the drinking water plus 2 g/kg acute dose was due to only one single high value which was more than four SD above the mean calculated from the other nine values determined in this experiment.

AFB_1 can be oxidized in a variety of positions[24]. Only one of these possibilities, the epoxidation of the 2,3-double bond, leads to the formation of a reactive DNA-binding metabolite[23]. Our data indicate that a 10-day pretreatment with ethanol did not induce the respective enzyme activities in such a way as to change the concentration of the reactive AFB_1-2,3-oxide near the DNA after administration of a low dose of AFB_1.

As opposed to the inducing effect of chronic ethanol treatment on the metabolism of various xenobiotics[7], ethanol itself has been shown to be a competitive inhibitor of the metabolism of many aromatic and chlorinated hydrocarbons[25]. Our experiment C did not reveal any dose-dependent effect of a single dose of ethanol on the formation of DNA-binding metabolites of AFB_1 in liver. The slight reduction shown up to 2 g/kg cannot be taken as an indication for an effect because it would be difficult to explain why the highest ethanol dose used should be less effective if a competitive inhibition is assumed to be the basis for this observation.

Our results provide in vivo evidence that a putative cocarcinogenic activity of alcohol on aflatoxin-induced liver tumor induction at low dose levels is unlikely to be due to a modulation of the initial DNA damage and repair within 24 h. Other mechanisms of cocarcinogenic action, such as cytotoxicity and the regenerative processes elicited[26-28], changes in DNA repair or alcohol-mediated genotoxicity via aldehydes and oxygen radicals[5] might play a more important role than changes of enzymatic activities. This lack of an observable effect, shown both for single and repeated alcohol administrations, contrasts with some positive results reported for nitrosamines[7-9]. The discrepancy shows that the modulations are probably specific for the structure of the carcinogen[11], for the target organ[13], and for the dose levels used both of carcinogen and of ethanol[12]. Any one single experimental system will therefore not be able to describe in full the complex role of alcohol in chemical carcinogenesis.

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Immunocytochemical demonstration of calmodulin in cells secreting by exocytosis

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Summary. Calmodulin is a regulator of several calcium-dependent cellular processes. It has been suggested that it plays a role in the mechanism of secretion. Employing an indirect immunoperoxidase technique at the light microscope level, this study demonstrates the presence of calmodulin in several exocytotic cells (mast cells, thyroid follicular cells, neurohypophyseal neurosecretory terminals, pancreatic β-cells and pancreatic acinus cells) in rat and man. The positive staining reaction for calmodulin was granular and at least in the case of rat mast cells it appeared to be associated with the granule membrane.
Key words. Immunocytochemistry; calmodulin; secretory granules.

Many intracellular processes, including stimulus-secretion coupling, are known to be calcium-dependent. One of the modulators of these processes is probably the troponin C-related protein, calmodulin (148 amino acid residues, mol.wt around 17,000) which appears to be ubiquitous in eukaryotic cells[1,2]. Calmodulin could play a role in stimulus-secretion coupling at several steps, as the protein is involved in cyclic AMP metabolism, arachidonic acid metabolism, activation of the actomyosin system, control of microtubule assembly and calcium-magnesium ATP-ase activity[3,4].

In indirect pharmacological studies and using radioimmunoassays and phosphodiesterase activation assays, the presence of calmodulin has been demonstrated in exocytotic cells: mast cells[4,5], neurohypophyseal neurosecretory terminals[6–9], follicular cells of the thyroid[10,11], pancreatic β-cells[12,13] and acinus cells of the exocrine pancreas[14].

In the present study, the indirect immunoperoxidase technique was employed to investigate the subcellular distribution of calmodulin in the above-mentioned cells.

Material and methods. Reagents and buffers. Calmodulin was purified from bovine brain according to Watterson et al.[15]. Polyclonal rabbit antiserum to calmodulin was prepared according to Slaninova and Thorn[9]. The antibody showed no cross-reactivity with troponin. The buffer used for the radioimmunoassay contained 1 mM EGTA. Measurements of calmodulin concentration by means of radioimmunoassay and by the phosphodiesterase activation method gave the same results in rat brain homogenates, bovine neurosecretomes and neurosecretory granules respectively. Normal goat serum, biotinylated goat antirabbit serum and avidin peroxidase were purchased from Vector. A Tris/HCl buffer, 50 mM, pH 7.4, containing 0.5 M NaCl and 0.01% of the detergent Nonidet P-40 was used for washing. Reagents were diluted in phosphate buffered saline containing 1% bovine serum albumin.

Tissue. Rat mast cells were obtained by peritoneal and thoracic cavity lavage, purified by Ficoll (Pharmacia) density gradient separation, washed and suspended in a Tris/HCl buffer[16]. Smears were prepared following resuspension in plasma. The smears were air-dried and fixed in methanol for 30 min.

Specimens from rat and human neurohypophysis, thyroid and pancreas were formalin fixed, paraffin-embedded and cut in serial sections. Mast cells were found in the loose connective tissue of these specimens.

All the paraffin-embedded sections were deparaffinized prior to immunostaining.

Immunostaining procedure. All steps apart from the primary antibody incubation were carried out at room temperature. The slides were treated with methanolic hydrogen peroxide 0.5% for 15 min followed by normal goat serum 1:50 for 15 min to block endogenous peroxidase activity and reduce background staining. They were incubated at 4 °C with anti-calmodulin 1:1000 for 72 h, with biotinylated goat antirabbit serum 1:200 for 35 min and with avidin peroxidase 1:400 for 50 min. Calcium concentrations were controlled using defined amounts of EGTA and calcium cloride in the incubation buffers. Staining was achieved by incubating the slides for 15 min with 0.5 mg diaminobenzidine per ml Tris/HCl buffer, with the addition of hydrogen peroxide for the last 8 min. Finally, the slides were dehydrated and mounted in DPX.

Control trials were performed employing non-immunized rabbit serum 1:1000 in place of anti-calmodulin. Moreover, the experiments were carried out following absorption of antibody with purified calmodulin.