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The DNA damaging drug cyproterone acetate causes gene mutations and induces glutathione-S-transferase P in the liver of female Big Blue™ transgenic F344 rats

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The gestagenic and antiandrogenic drug cyproterone acetate (CPA) is mitogenic, tumorigenic and induces DNA-adducts and DNA-repair synthesis in rat liver. Thus CPA is expected to be mutagenic. However *in vitro* mutagenicity test systems were negative. To examine whether CPA induces mutations in rat liver, the *in vivo* mutation assay based on Big Blue™ transgenic F344 rats was employed. Single oral doses of 25, 50, 75, 100 and 200 mg CPA/kg b.w. respectively were administered to female Big Blue™ rats. Six weeks after treatment, liver DNA was assayed for mutations. At the highest dose, 200 mg CPA/kg b.w., the frequency of $(17 \pm 4) \times 10^{-6}$ spontaneous mutations was increased to a maximum of $(80 \pm 8) \times 10^{-6}$ mutations. One-hundred and 75 mg CPA/kg b.w. resulted in mutation frequencies of (35 ± 5) and $(27 \pm 5) \times 10^{-6}$, respectively. The mutation frequency at doses of 50 and 25 mg CPA/kg b.w. was similar to that of vehicle treated controls. Statistical analysis of the dose-effect relationship revealed that it was not possible to decide whether a threshold dose exists or not. DNA adducts were analyzed by the ^{32}P-postlabelling technique. The total level of the major and the two minor adducts observed in the autoradiograms increased between doses of 25 to 75 mg CPA/kg b.w. to a maximum of ~12 000 ± 3000 adducts per 10^9 nucleotides. The level did not further increase significantly with 100 and 200 mg CPA/kg b.w. After CPA treatment no preneoplastic liver foci were observed. However, single glutathione-S-transferase placental form (GST-P) positive hepatocytes were observed and the frequency was dependent on the dose. These cells are not supposed to represent initiated cells, since they occurred only transiently after 6 weeks and disappeared thereafter completely. In conclusion, our results demonstrate that CPA is mutagenic *in vivo*. The mutation frequency increased at high CPA doses, when the increase of the DNA adduct formation had already ceased. This suggests that the mitogenic activity of CPA is required to express the mutations.

***Abbreviations:** ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3.); CPA, cyproterone acetate; ENU, N-ethyl-N-nitrosourea; GGT, γ-glutamyl-transpeptidase (EC 2.3.2.2.); glutathione-S-transferase placental form (GST-P), glutathione-S-transferase placental form (EC 2.5.1.18.); HSST, hydroxysteroid sulfonyltransferase (EC 2.8.2.); X-Gal, 5-bromo-4-chlor-3-indoloyl-β-D-galactopyranoside.

Introduction

Cyproterone acetate (CPA*) is a synthetic progesterone analogue with antiandrogenic and progestogenic activity. The compound is widely used in the therapy of androgenisation symptoms in women and, at high doses, for the treatment of prostate cancer and the suppression of sexual drive in men (1). CPA induces the formation of tumors in rat liver after long term feeding of high doses (2).

Subsequently, the drug was shown to exhibit genotoxic activity in rat liver. CPA causes CPA specific DNA adducts and elicits DNA repair synthesis in the hepatocytes of rats (3,4) and humans (5–7). Hepatic DNA adducts formed *in vivo* persist for several months (8). Furthermore, CPA induces preneoplastic liver foci and is a putative tumor promotor in rats (9,10). Although these findings indicate that CPA is a complete carcinogen, attempts to probe for its mutagenic activity *in vitro* failed. The drug did not induce mutations in the Ames *Salmonella/*microsome test and the HGPRT test in V79 cells (11).

An alternative method to detect the mutagenic effects is provided by the commercially available Big Blue™ transgenic rat mutation assay. Following *in vivo* treatment with CPA, the target gene is recovered from the tissues of interest and screened for mutations. The suitability of this method has been demonstrated recently (12,13).

The present investigation was designed to study the dose effect relationship of mutation frequency and of DNA adduct formation in the liver as a causative event after administration of CPA in various doses. Another goal was to examine whether the mutations lead to initiated hepatocytes and to preneoplastic foci, as early precursors of liver tumors. Liver sections were screened histochemically for the emergence of enzyme-altered preneoplastic foci. Thus our experimental strategy allows us to study subsequent steps in tumorigenesis, i.e. the formation of DNA adducts, the induction of mutations and the occurrence of preneoplastic lesions.

Materials and methods

Chemicals

The following chemicals were purchased from suppliers in Germany: cyproterone acetate, N,N-dimethylformamide, micrococcal nuclease, potato apyrase (grade III) and ribonuclease T1. Anti-rabbit IgG-peroxidase was purchased from Sigma-Aldrich, Deisenhofen; olive oil was from Fluka, Neu-Ulm; Spleen phosphodiesterase and proteinase K was from Boehringer, Mannheim; RNase A was from Serva, Heidelberg; T4 polynucleotide kinase was from Amersham, Braunschweig; PEI-cellulose TLC sheets, 0.1 mm, were from Macherey-Nagel, Düren; [γ-^{32}P]adenosine 5′-triphosphate, tetra-(triethylammonium) salt, 3000 Ci/mmol, was from NEN-DuPont, Köln; the Transpack packaging extract was from Stratagene, Heidelberg; 5-bromo-4-chlor-3-indoloyl-β-D-galactopyranoside (X-Gal) was from Biosynth, Staad, Switzerland; rabbit anti-rat GST-Yp was from Biotrin Int., Dublin, Ireland. All materials and reagents were of microbiological or analytical purity.

Animals

Homozygous female transgenic Fischer 344 rats (Big Blue™ Rat) were obtained from Stratagene, La Jolla, USA when 2 months of age and housed two per cage. The animals were feed a standard diet (Altromin) and water *ad*

libidum. The dark/light cycle was 12 h/12 h. Each cell of the rat carries 30–40 copies of the lambda LacI-α LacZ shuttle vector (lambda LIZ).

Treatment

Experiment 1. This experiment was performed with a small number of animals to find out whether single doses of CPA raise the overall mutation frequency and to determine the appropriate expression time. Eight animals, 3 months old, were divided into four groups of two rats, each receiving orally single doses of 25, 50 and 100 mg CPA/kg b.w. dissolved in 2 ml olive oil/kg b.w. or the vehicle only. One animal from each group was killed after 11 weeks and 22 weeks. Liver samples were processed as described below.

Experiment 2. This experiment was performed with 35 animals, 8 months old, divided into seven groups of five animals each to demonstrate the dose–response effect of CPA. One group served as a vehicle treated control, another one as a positive control with N-ethyl-N-nitrosourea (ENU, 1×80 mg/kg b.w. i.p.) as a mutagenic agent. The animals of the other five groups each were treated orally with single doses of 25, 50, 75, 100 and 200 mg CPA/kg b.w. respectively, dissolved in 2 ml of olive oil/kg b.w. Six weeks later all animals were killed by cervical dislocation under ether anesthesia.

The livers were quickly removed, frozen in liquid nitrogen and stored at –80°C until DNA preparation. For histological examination pieces of the left and middle liver lobes were frozen at –70°C for cryostat microtomy or fixed in buffered formaldehyde and embedded in paraffin.

Mutation assay

The mutation frequency of the LacI transgene was determined according to the protocol of Dycaico *et al.* (12). Briefly, the rat liver genomic DNA was isolated with Qiagen™ genomic tips following the manufacturers protocol. The lambda LIZ shuttle vector containing the LacI target gene was removed and packaged with envelope proteins to produce infectious lambda phages using the Stratagene™ packaging extract according to the manufacturers protocol. *Escherichia coli SCS8* bacteria were transformed and plated on X-Gal containing agar plates. Mutant $LacI^-$ (blue) and wild type $LacI^+$ (white) plaques were scored and the mutation frequency (mutant/[mutant + wild type]) was determined.

Determination of DNA adducts by ^{32}P-postlabelling

DNA was isolated and purified from liver tissue homogenates by using RNase- and proteinase K treatment followed by phenol extraction as described (14). DNA concentration was determined fluorometrically using bisbenzimide and calf thymus DNA as a standard (15). The butanol enrichment procedure was employed (16) with minor modifications (3). The dried butanol extracts were labeled with [γ-^{32}P]ATP and treated with apyrase to degrade excess ATP. PEI cellulose thin layer chromatography was performed according to Topinka *et al.* (3). After washing and drying, the sheets were autoradiographed at –80°C for 4–96 h depending on the presumed amount of radioactivity. The radioactive spots on the chromatogram were cut out and the radioactivity was determined by scintillation counting. All values were corrected for the background level.

Histochemistry

Cryostat sections were cut and stained to assay the activity of adenosine-5′-triphosphatase (ATPase) and γ-glutamyltranspeptidase (GGT) as described earlier (17). Sections from formalin-fixed, paraffin-embedded liver tissue were taken for the detection of glutathione-S-transferase placental form (GST-P) by indirect immunoperoxidase staining. The sections were stained using rabbit anti-rat GST-Yp (diluted 1:400) as primary antibody. Anti-rabbit IgG-peroxidase (diluted 1:400) was used as secondary antibody. Peroxidase activity was determined with diaminobenzidine as substrate. GST-P expressing single cells were counted from six sections per dose group. A total field of ~0.5 cm^2/group was measured.

Statistical methods

Four dose–response models were considered in order to summarize the data on mutation frequencies:

1. Linear model: $p = a + b \cdot dose$
2. Linear quadratic model: $p = a + b \cdot dose + c \cdot dose^2$
3. Linear E-NOEL model: $p = a$ for dose $\leq c$; $p = a + b \cdot (dose - c)$ for dose $\geq c$
4. Logit model: $p = a + (1 - c) \cdot F(a + b \cdot \ln dose)$

(p = expected relative mutation frequency)

The observed relative mutation frequency was taken to be the total number of mutant plaque forming units divided by the total number of plaque forming units screened for all animals treated with the same dose. The responses were assumed to follow a binomial distribution. Possible overdispersion was examined by computing the replication error following McCullagh and Nelder (18). An estimate of ~1.0 justified the assumption of binomial variation.

Estimates of the model parameters a, b and c were obtained using maximum-likelihood methods. Models 1 and 2 were fitted as Generalized Linear Models with binomial error distribution and identity link using SAS software, procedure GENMOD (19). Model 3 was fitted as a binomial non-linear regression model with identity link minimizing the binomial log-likelihood using SAS procedure NLIN (20). Model 4 was fitted as a threshold tolerance model using SAS procedure PROBIT (20). Overall goodness of fit was assessed by using the deviance statistic which was assumed to follow a chi-square distribution. Having excluded the possibility of overdispersion this statistic will reflect inadequacies of the systematic part of the fitted models. For more detailed assessment standardized deviance residuals were used. All statistical tests were carried out at a nominal 95% confidence level. The graph of the fitted models in Figure 1 was produced employing SAS/GRAPH software (21).

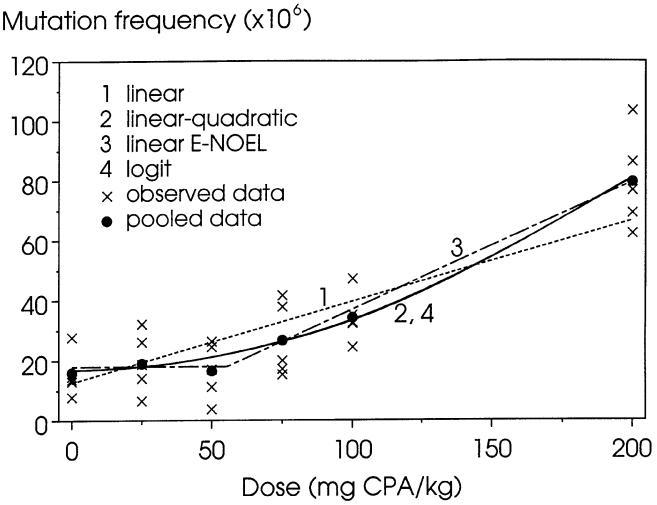

Fig. 1. Plot of the predicted proportion of mutants versus dose of CPA for four fitted dose–response curves. Included are observed (×) and pooled (●) data points.

Results

Mutagenesis assay

The experiments performed in this study reveal that CPA was a mutagenic compound in rat liver. In experiment 1, performed with one animal per dose and expression periods of 11 and 22 weeks, a dose of 100 mg CPA/kg b.w. was found to increase the mutation frequency determined in the controls by a factor of almost 4, while doses of 25 and 50 mg CPA/kg b.w. were ineffective, with the possible exception of 50 mg CPA/kg b.w., 11 week assay time (Table I).

Experiment 2 performed with five animals per dose group and an expression period of 6 weeks revealed a dose dependent increase of mutation frequency at doses between 75 and 200 mg CPA/kg b.w. (Figure 1, Table I). Mutation frequencies determined at doses of 25 and 50 mg CPA/kg b.w. did not exceed those determined in control animals. For interpretation four common dose–response models were employed to fit the data.

For the 6 week assay, Figure 1 presents a plot showing the observed proportions of mutants of the individual rats, the pooled proportions and the four dose–response models fitted. The linear model (Model 1) produced the worst fit to the data. Models 2 and 4 are better than Model 1 and indistinguishable from each other. The linear E-NOEL model (Model 3) fits the data even slightly better than Models 2 and 4. The overall lack-of-fit statistics support these observations. For Model 1 the deviance has an associated *p* value of 0.03 indicating

Table I *LacI* mutation data from the livers of female lambda/*LacI* rats exposed to CPA

Expression period[a]	Treatment[b]	Number of animals	Summarized pfu[c]	Summarized mutant pfu[d]	Mutant frequency[e]	± SD
6	0	5	1370600	23	16.8	3.5[f]
6	25	5	1212200	24	19.8	4.0[f]
6	50	5	1155500	20	17.3	3.9[f]
6	75	5	1203100	33	27.4	4.8[f]
6	100	5	1201700	42	34.9	5.4[f]
6	200	5	1195200	96	80.3	8.2[f]
11	0	1	275100	8	29.1	10.3[g]
11	25	1	336400	10	29.7	9.4[g]
11	50	1	298800	13	43.5	12.1[g]
11	100	1	290900	32	110.0	19.5[g]
22	0	1	300000	8	26.7	9.4[g]
22	25	1	310800	8	25.7	9.1[g]
22	50	1	247300	7	28.3	10.7[g]
22	100	1	249850	24	96.1	19.6[g]

[a]Number of weeks between oral treatment and euthanasia/tissue collection.
[b]Single oral dose (mg CPA/kg b.w.).
[c]Summarized number of pfu screened.
[d]Summarized number of mutant pfu.
[e]Mutant frequency is expressed as the summarized mutant pfu per million pfu screened.
[f]Binomial standard deviation ($\times 10^{-6}$).
[g]Methodic standard deviation ($\times 10^{-6}$).

statistically significant evidence against the hypothesis of no lack-of-fit. The *p*-values for the remaining Models 2, 3 and 4 are very high indicating little evidence against the no lack-of-fit hypothesis ($p = 0.74$; 0.91; 0.75, respectively). For the linear model one also finds that three of six residuals (at dose = 0, 50, and 100) indicate statistically significant lack-of-fit. In addition, in the linear-quadratic model the quadratic term is highly significant ($p = 0.003$). In summary then, the linear model appears to be inacceptable, contrary to the rest of the models.

Determination of DNA adducts

DNA adduct analysis was performed on the same liver samples as used for the mutation assay. The DNA fingerprints reveal a pattern of one major adduct (A) and two minor adducts (B and D) known from previous studies with female rats (3). The total adduct level showed a dose-dependent increase at doses of 25, 50 and 75 mg CPA/kg b.w. respectively, 6 weeks after administration (Figures 2 and 3). Higher doses of 100 and 200 mg CPA/kg b.w. respectively, did not further increase the total adduct level. Eleven and 22 weeks after CPA dosing, the adduct levels were found to be lowered by ~30% and 50% respectively, compared to those determined after 6 weeks.

Histochemical demonstration of enzyme alteration

Liver sections were screened histochemically for preneoplastic foci. No enzyme altered preneoplastic liver foci were observed 6, 11 and 22 weeks after administration of CPA, using ATPase, GGT and GST-P as markers.

Treatment with CPA induced numerous single GST-P positive hepatocytes, 6 weeks after treatment with CPA. These cells were mainly arranged around the central veins (Figure 4). The number of GST-P positive hepatocytes was dependent on the dose. In untreated control animals <10 cells/cm^2 liver section were found. Doses of 25, 50 and 75 mg CPA/kg b.w. respectively evoked ~800–1000 GST-P positive cells/cm^2. A 5- to 15-fold increase of the number of GST-P positive cells was observed with 100 and 200 mg of CPA/kg b.w. as compared to the lower doses, the GST-P positive cells forming dense clusters around the central veins (Figure 4C and D). Eleven and 22 weeks after CPA treatment, no GST-P positive cells were found.

Discussion

Since it is known that the synthetic progestin CPA induced DNA repair synthesis (4), persistent DNA adducts (3,8), preneoplastic foci (9) and tumors in rat liver (2), the compound was suspected to possess a mutagenic potential. The principle finding of the present study is that CPA enhances the frequency of mutations in livers of female transgenic Big Blue^{TM} rats in a dose-dependent manner, indicating that CPA is mutagenic. This finding and previous studies on the pathway of bioactivation of CPA provide a rationale to explain why CPA did not induce gene mutation in two frequently used *in vitro* mutation assays: the Ames *Salmonella*/microsome test and the HGPRT test in V79 cells (11). Most likely, CPA was not activated to mutagenic metabolites, because these systems are not equipped with appropriate enzymes and cofactors. Recent findings indicate that hydroxysteroid sulfotransferase (HSST) is involved in the ultimative step of activating CPA to DNA-binding metabolites (22). The enzyme converts 3-hydroxy-CPA formed by reduction of CPA to a labile 3-hydroxysulfate ester, which spontaneously eliminates the sulfate group, thereby forming a DNA binding carbonium ion. HSST is not known to occur in the two assay systems.

The dose effect curve exhibited a discontinuous form. At higher doses, CPA caused a significant linear increase of the mutation frequency, while the two lower doses of 25 and 50 mg CPA/kg b.w. respectively did not significantly elevate the mutation frequency observed in control animals. The three statistical models found to fit the experimental data allow two interpretations. On one hand, the finding that the linear E-NOEL model (Model 3) was able to fit the data can be interpreted in favour of the existence of a threshold dose, which lies in the range of ~50 mg CPA/kg b.w. On the other hand the linear quadratic model (Model 2) and the logit model (Model 4) fit the data equally well providing no evidence for the existence of a threshold dose but indicate the mutagenic

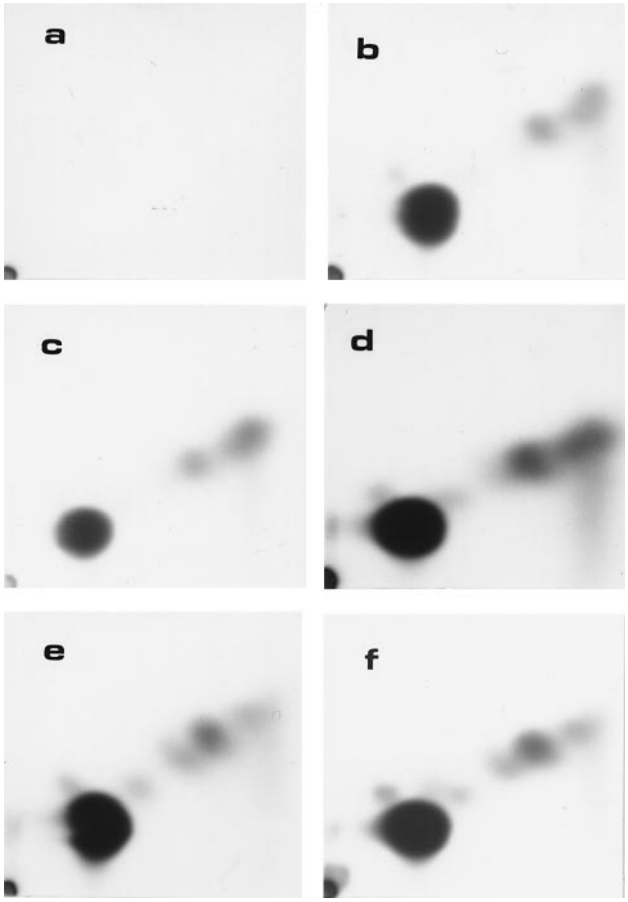

Fig. 2. Autoradiograms of ^{32}P-postlabelled hepatic DNA adducts from animals treated with CPA as described in Materials and methods under experiment 2. The autoradiograms shown are derived from one animal each treated with a single dose of (**a**) the vehicle, (**b**) 25 mg, (**c**) 50 mg, (**d**) 75 mg, (**e**) 100 mg and (**f**) 200 mg CPA/kg b.w., respectively.

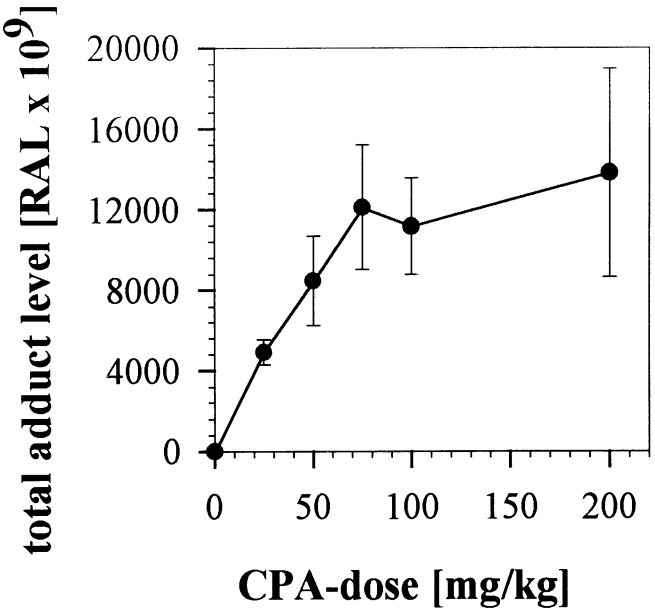

Fig. 3. Dose dependence of CPA derived DNA-adduct levels in the liver of female Big Blue^{TM} F344 rats. The figure shows the mean values/SD of the total level of all detectable adducts of five animals each.

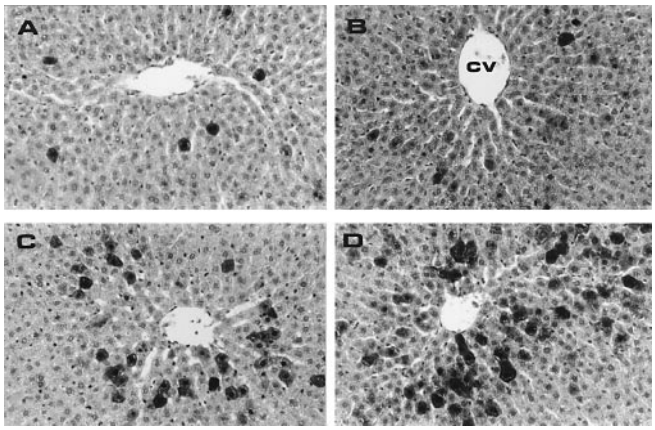

Fig. 4. Incidence of GST-P-positive hepatocytes (dark strain) around the central veins (cv) dependent on the dose of CPA (magnification ×170). (**A**) 25 mg CPA/kg b.w.; (**B**) 75 mg CPA/kg b.w.; (**C**) 100 mg CPA/kg b.w.; (**D**) 200 mg CPA/kg b.w.

effectiveness to be reduced in the low dose range. Although Model 3 fits the data slightly better than Models 2 and 4, it is impossible to discriminate between the two interpretations.

The dose dependence of the DNA adduct levels showed a linear increase for low doses up to 75 mg CPA/kg b.w., while at the high doses of 100 and 200 mg CPA/kg b.w. respectively, the adduct levels did not further increase. This may be the result of a saturation of HSST, the CPA-activating enzyme, or due to a shortage of a cofactor required. Since sulfonylation is rate limiting for CPA bioactivation, it is reasonable to assume that HSST is saturated with 3-hydroxy-CPA at high doses of CPA. Shortage of the cofactor 3′-phosphoadenosine-5′-phosphosulfate is less likely to occur, since it is readily resynthesized when consumed (23).

Obviously, the dose effect curve of CPA induced mutations does not coincide with the dose effect curve of CPA-DNA adduct levels. The mutation frequencies increased within a dose range where DNA adduct level curve had already reached a plateau. We assume that an additional effect of CPA operating at high CPA doses only, probably the mitogenic activity, is required to express the mutations. An increase in the hepatic RNA and DNA synthesis, mitotic rate and liver growth was found in female rats when CPA was administered in six consecutive oral doses between 40 and 100 mg/kg b.w. (10). A single dose of 100 mg CPA/kg b.w. strongly induced replicative DNA synthesis in hepatocytes of female rats (24).

Another biological marker of genotoxicity, the induction of enzyme-altered preneoplastic foci, was not affected. In contrast, in an earlier study with rats CPA exhibited an initiating potency. Preneoplastic liver foci emerged in a dose-dependent manner. However, in that experiment a promoting stimulus had been employed additionally, which favoured the development of foci (9). Apparently, a single dose of CPA without promoter treatment is not sufficient to evoke foci.

A striking observation, however, was the dose-dependent induction of GST-P in single hepatocytes. These cells accumulate in dense clusters mainly around the central veins, clearly distinguished from foci. Only a marginal number of GST-P positive cells were seen in controls. GST-P is a valuable marker of preneoplastic foci, nodules and tumors (25). It is not expressed in normal hepatocytes (26). Consequently single GST-P positive hepatocytes occurring after treatment with carcinogens are suggested to be initiated cells (27). Though

we cannot definitely exclude the existence of initiated cells in our study, the GST-P positive hepatocytes observed here are not supposed to represent initiated cells. They occur temporarily, 6 weeks after treatment but not after 11 and 22 weeks. Similarly, a transient emergence of GST-P positive hepatocytes in rats has been reported after treatment with various non-genotoxic chemicals, e.g. the coffee specific diterpenes cafestol and kahweol (28), the mitogenic agents lead acetate and lead nitrate (29), as well as ethoxyquin (30) and the antioxidants, butylated hydroxytoluene and butylated hydroxyanisole (31). The transient induction of GST-P may rather reflect an adaptive response to a toxic damage by CPA since the enzymes of the GST family detoxify electrophilic xenobiotics by catalyzing their conjugation with glutathione.

In conclusion, the present results clearly indicate a dose-dependent mutagenic potential for CPA. Thus, the gap between the known activity of CPA to damage DNA in rat liver, on one hand, and to induce preneoplastic cell lesions and tumors, on the other hand, is closed and CPA now fulfils the criteria of a complete carcinogen. However, at present it is premature to speculate about the significance of our findings regarding the health risk possibly associated with a long term therapy with CPA. It has been shown that CPA induces DNA adducts in human hepatocyte cultures at levels between 5 and 25% of those determined in rat hepatocytes (6,7) and in human liver slices at levels between 25 and 100% of those found in rat liver slices (5). Moreover, it is known that repeated administration of small doses of CPA causes an almost linear accumulation of DNA adducts in rats (8). Therefore, long term therapy with daily doses of 2 and 10 mg taken by women or with doses of 100 and 300 mg repeatedly applied to men likely causes high DNA adduct levels that may be similar to those causing mutations in rat liver. Because we do not know whether and to what extent the CPA adducts formed are converted into gene mutations in human liver, it is impossible at present to assess the mutagenic risk that may be associated with long term therapy of CPA.

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