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Hepatocarcinogens induce gene mutations in rats in fibroblast-like cells from a subcutaneous granulation tissue

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Gene-mutations at the 6-thioguanine locus, in fibroblast like-cells rapidly proliferating on the inside of rat subcutaneous air pouches were analysed (Granuloma Pouch Assay). Target cells were exposed directly by injection into the air pouch, or systemically by oral or intraperitoneal administration to three hepatocarcinogens aflatoxin B_1, 2-acetylaminofluorene (2-AAF) and vinylchloride. In addition ethylnitrosourea (ENU) was used as a positive control, and 2-aminofluorene to characterize the pharmacokinetic behaviour of 2-AAF. When administered directly, all chemicals except 2-AAF induced a dose-dependent increase in gene-mutation frequencies. However, 2-AAF was mutagenic after oral administration. The highest inducible mutation frequencies found with the hepatocarcinogens were 8 to 10 times the spontaneous mutation frequency, but only ~10% of that found with the control mutagen ENU. The known enzymic activation capacity of the granulation tissue, and the mutagenic activity of other chemicals in this system already reported, suggest that after direct exposure, the prostaglandin H synthetase pathway or lipoxygenases are involved in the activation of the hepatotoxins to gene mutation inducing species *in vivo*. 2-AAF seems to be converted via stable intermediates to the ultimate mutagenic species in the extra-hepatic target tissues.

Introduction

Risk evaluation of chemicals must take into account their gene-mutation inducing capacity in extrahepatic tissues *in vivo*. Not only because the first interaction of a chemical with an organism occurs at extrahepatic tissues (e.g. via inhalation, skin contamination or ingestion) but also, because after systemic exposure stable intermediates may be formed for instance in the liver, which are mutagenically active in extrahepatic tissues (33). The analysis of gene-mutations is important because a chemical's gene-mutation inducing activity does not necessarily correlate with its clastogenic activity e.g. determined in bone marrow.

Chemicals often not considered hazardous in extrahepatic tissues include those known to be activated by cytochrome P-450 dependent mono-oxygenases. In tissues with low or zero mono-oxygenase activity, however, the peroxidative activation of xenobiotics might provide an alternative activation pathway. In rats, this pathway has been reported to play a role in the lung, renal medulla, forestomach, bone marrow, urinary tract and granulation tissue (28). Its role in the conversion of chemicals to gene-mutation inducing metabolites *in vivo* needs to be elucidated.

The method of choice for studying this problem is the Granuloma Pouch Assay. In this animal model, among other genetic endpoints, gene-mutations can be detected in individual, *in vivo* exposed cells (34). Target cells can be exposed directly to test compounds including gaseous chemicals. This feature is a unique characteristic of this assay not present in other established *in vivo* mutagenicity tests with extrahepatic tissue such as blood cells (41), bone marrow cells (31,46), embryonic cells (17) or germ cells (14).

In the Granuloma Pouch Assay, no cytochrome P-450 dependent mono-oxygenases have yet been detected in the target tissue. The prostaglandin H synthetase (28) and the lipid-peroxidation dependent pathways (29), however have been identified. Xenobiotics might serve as reducing factors during the peroxidative reduction of prostaglandins ($PGG_2 \rightarrow PGH_2$) (42). *In vitro*, it has been shown that a number of chemicals can be cooxidized by this pathway into reactive intermediates (15) or mutagenic metabolites (45).

Three of the five chemicals tested, aflatoxin B_1 (AFB_1*), 2-acetylaminofluorene (2-AAF) and gaseous monomeric vinyl-chloride (VCM) were carcinogenic predominantly in liver. VCM induces angiosarcoma in laboratory animals and man (16,18). *In vitro*, AFB_1 and aromatic amines are known substrates for the PHS pathway (6,7). 2-Aminofluorene (2-AF) was included in this study to assist in the elucidation of the pharmacokinetics of 2-AAF, and ethylnitrosourea (ENU) was used as a positive control.

Thus, by comparing the mutagenic activity detected after systemic application and after direct exposure, the pharmacokinetic behaviour of a mutagenic species can be characterized (36).

Materials and methods

Animals and target cells

Randomly bred male albino Sprague–Dawley rats (SIV 50 Ivanovas Kisslegg, FRG, 230–260 g, 54 ± 4 days old) were used. Aliquots of 25 ml of germ free air were injected subcutaneously at the midpoint of the scapular area on the back of the animals. This induced sufficient growth of cells forming the target tissue at the inside wall of the air pouch: additional application of croton oil would not have produced more fibroblast-like cells capable of growing *in vitro* (34,35). The test compound was administered at the height of proliferative activity (48 h after pouch formation), either intraperitoneally, directly into the air pouch or by gavage.

Two days later, the animals were sacrificed using CO_2. The granulation tissues were removed by aseptic dissection. Red blood cells were lyzed by washing the tissue for 10 s in sterile distilled water. The tissue was then minced in 1 ml Dulbecco's phosphate buffer (PBS, pH 7.4), and subjected to enzymic dissociation [10 ml PBS containing collagenase Type I (600 Mandl-U) and Dispase II (8U), stirring for 45 min at 35°C]. Subsequently, the resultant suspension was filtered through two layers of Kodak lens paper: the filtrate, consisting of tissue fragments, was then minced and incubated for a second time as described above. Finally, the two cell suspensions were pooled, centrifuged for 5 min at 134 *g* and resuspended in culture medium [Dulbecco's modified Eagle medium, 10% fetal calf serum (FCS), 50 μg/ml Gentamycin]. The viability of cells, as determined by the trypan blue exclusion test was above 95%, except at the highest exposure levels, where the number of cells without damaged membranes dropped below

*Abbreviations: AFB_1, aflatoxin B_1; 2-AAF, acetylaminofluorene; 2-AF, 2-aminofluorene; VCM, vinylchloride, monomeric; ENU, ethylnitrosourea; FCS, fetal calf serum; IQ, 2-amino-3-methylimidazo[4,5-*f*]-quinoline; BP, benzo[*a*]-pyrene; HGPRT, hypoxanthine-guanine phosphoribosyl transferase.

Table I. Mutation induction *in vivo* at the 6-TG locus in fibroblast-like cells of the subcutaneous granulation tissue in rats

Chemical exposure level	Cloning efficiency I CE (mean ± SD)	Cloning efficiency II CE (mean ± SD)	No of clone forming cells tested ($\times 10^6$)	Mutant frequency (mean ± SD) (10^{-6})	No. of animals tested
Control I	14.5 ± 4.6	64.9 ± 14.4	6.800	2.48 ± 3.69	9
ENU					
Application into the air pouch:					
0.06 mg/GP	20.8	75.5	0.906	27.60[a]	1
0.25 mg/GP	18.5	92.7	1.112	111.50[a]	1
0.33 mg/GP	4.3	12.0	0.144	340.20[a]	1
1.33 mg/GP	12.9	89.0	1.068	148.80[a]	1
Intraperitonal application:					
20 mg/kg i.p.	25.5 ± 6.0	59.1 ± 16.8	2.097	60.7 ± 15.3[a]	3
80 mg/kg i.p.	20.6 ± 2.9	67.3 ± 19.0	2.422	280.9 ± 148.3[a]	3
Aflatoxin B_1					
Application into the air pouch:					
10 μg/GP	10.4	66.3	0.796	1.26	1
20 μg/GP	13.4 ± 1.0	67.5 ± 5.1	3.107	12.74 ± 12.65[a]	4
40 μg/GP	14.9 ± 6.2	49.9 ± 19.8	4.830	21.71 ± 13.03[a]	9
60 μg/GP	12.7	71.2	1.708	8.75[a]	2
Vinylchloride					
Application into the air pouch:					
0.5 mg/GP	12.4 ± 2.2	62.0 ± 5.6	2.645	4.20 ± 6.40[a]	4
1.0 mg/GP	13.1 ± 7.6	58.1 ± 9.1	2.092	17.04 ± 7.89[a]	3
2.0 mg/GP	15.4 ± 3.0	51.7 ± 7.4	2.982	10.86 ± 11.30[a]	5
4.0 mg/GP	19.6	50.1	1.050	9.55[a]	2
Mutation frequencies in subpopulations:					
Total cell population					
Control	7.6 ± 0.1	55.5 ± 6.2	1.818	0	3
VCM 2.0 mg/GP	18.5	50.0 ± 9.0	1.794	18 10 ± 7.60[a,b]	3
Fraction (1B+2)					
Control	18.5	50.0	1.200	0.70	2
VCM 2.0 mg/GP	23.6 ± 4.8	48.8 ± 12.3	1.632	28.50 ± 10.20[a,b]	3
Control II	16.9 ± 4.4	68.9 ± 17.6	12.202	2.97 ± 3.36	15
2-Acetylaminofluorene					
Application into the air pouch:					
0.8 mg/GP	16.9	72.5	1.145	1.41	2
1.6 mg/GP	13.5 ± 8.5	73.8 ± 3.9	2.505	7.39 ± 3.03[a]	3
3.2 mg/GP	16.8	64.2	1.260	0.72	2
14 mg/GP	12.0	59.7	0.716	0	1
20 mg/GP	15.4	22.7	0.272	0	1
Oral application:					
20 mg/kg p.o.	13.8	55.8	1.163	5.51	2
40 mg/kg p.o.	14.9 ± 6.0	53.2 ± 17.1	2.343	11.51 ± 11.10[a]	4
60 mg/kg p.o.	19.0 ± 6.6	65.6 ± 13.0	3.148	16.06 ± 12.67[a]	4
80 mg/kg p.o.	18.8 ± 7.4	63.3 ± 13.2	1.986	12.46 ± 13.19[a]	4
120 mg/kg p.o.	17.0	65.9	1.580	7.60[a]	2
Pretreatment 2× intraperitoneally, subsequently 1× oral application:					
3 × 20 mg/kg	28.1	88.6	2.037	8.16[a]	2
3 × 40 mg/kg	20.9 ± 3.5	75.8 ± 13.4	3.248	21.09 ± 17.01[a]	4
3 × 60 mg/kg	16.7 ± 2.7	71.7 ± 17.2	3.248	35.52 ± 29.10[a]	4
3 × 80 mg/kg	9.6	89.4	1.714	11.68[a]	2
2-Aminofluorene					
Application into the air pouch:					
0.1 mg/GP	22.6	48.2	0.578	3.42	1
0.2 mg/GP	13.3	44.5	0.225	0	1

Table I. continued

Chemical exposure level	Cloning efficiency I CE (mean ± SD)	Cloning efficiency II CE (mean ± SD)	No. of clone forming cells tested ($\times 10^6$)	Mutant frequency (mean ± SD) (10^{-6})	No. of animals tested
0.4 mg/GP	12.5	46.2	0.850	27.54[a]	2
0.8 mg/GP	18.1	83.5	2.004	36.95[a]	2
1.6 mg/GP	6.6	66.3	1.591	9.38[a]	2
3.2 mg/GP	no growth in vitro				1
Oral application:					
15 mg/kg p.o.	11.8	34.6	0.801	2.00	2
30 mg/kg p.o.	13.2	51.5	1.236	4.05	2
60 mg/kg p.o.	19.2	40.5	0.122	0	1
200 mg/kg p.o.	lethal within 48 h after application				1

[a]Significantly different from the corresponding control values at $P < 0.05$ one sided, according to Kastenbaum and Bowman (23).
[b]Significantly different in pairs.

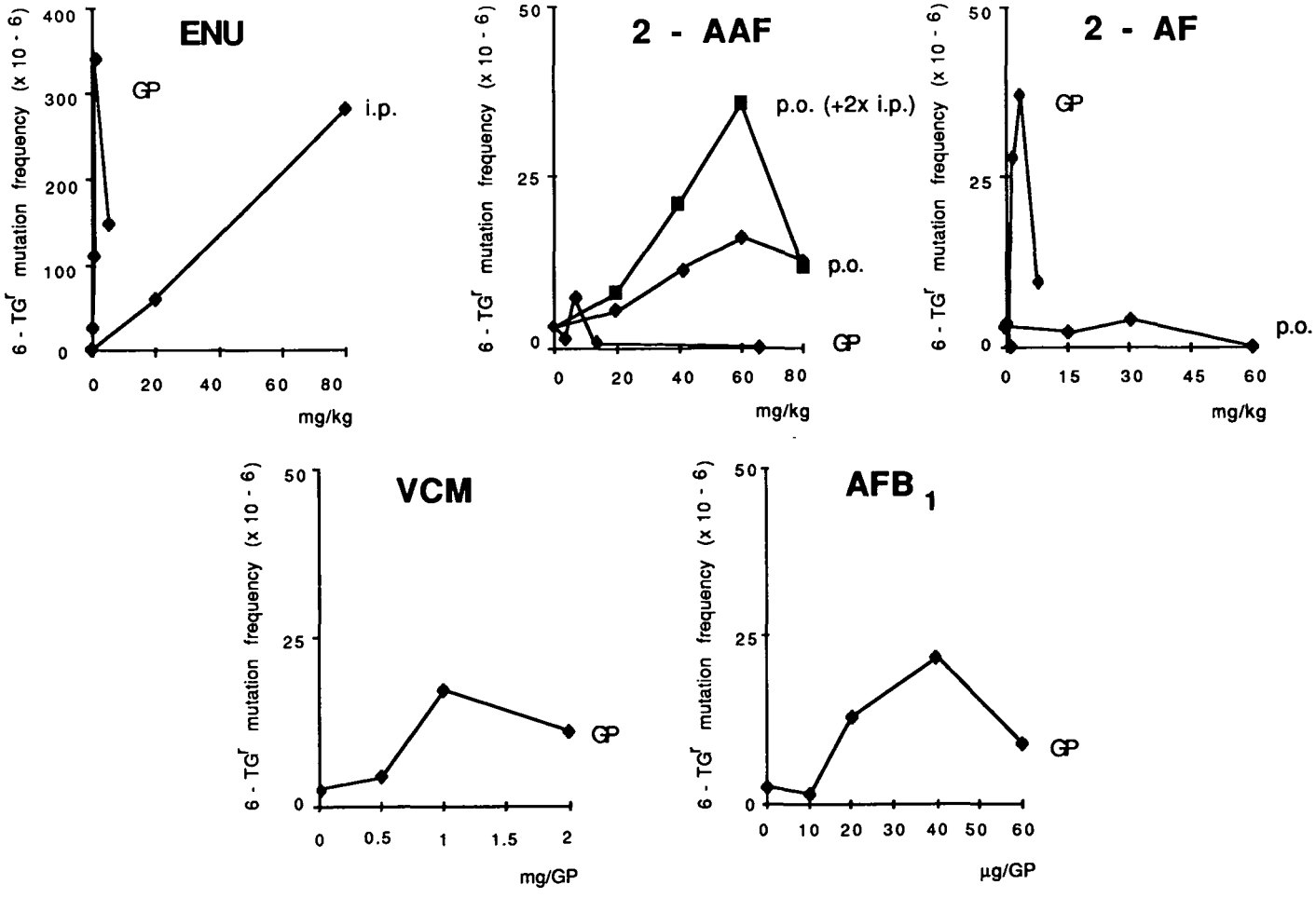

Fig. 1. Exposure-response curves of gene-mutation frequencies (6-TG^r) after oral application (p.o.), application into the air pouch (GP), or after intraperitoneal pretreatment (2 × i.p.).

90%. On average, 20.5 ± 7.6 × 10^6 (N = 26) nucleated cells were collected per rat.

Chemicals and exposure

Chemicals were injected into the air pouch or administered by gavage in a final volume of 1 ml. AFB_1 (Sigma, St Louis, MO, puriss. >99.5% analyzed by GC) was dissolved in ethanol. The concentrations were determined spectrophotometrically at 360 nm before dilution (EtOH <4%, v/v) with culture medium without FCS. 2AAF (Fluka, Buchs, Switzerland, puriss. 98% UV) was dissolved in polyoxyethylated castor oil (Mugolfen 620, GAF Cooperation, Zug, Switzerland). The exposure level of VCM (Fluka, Buchs SG, Switzerland) was analyzed by gas chromatography (2 m glass column, inner diameter 2 mm, Chromabsorb GAW, 80/100 mesh, 5% SP-100, T injection 200°C, T column 21°C, T detector 300°C, detector FID, carrier gas N_2, 46 cm^3/min), samples being taken from the pouch immediately after administration. 2-AF was dissolved in ethanol and diluted in culture medium without FCS. ENU was dissolved in 0.9% saline solution. Administration routes, sequence and exposure levels are summarized in Table I.

Cell culture and selection of mutants resistant to 6-thioguanine

3 × 10^3 (10^3 cells/100 mm dish) of the freshly isolated cells were tested for their primary cloning efficiency (I CE) after incubation for 7 days. 10^6 cells were cultured for 3 days in a 150 mm dish, trypsinized and incubated for another 3 days.

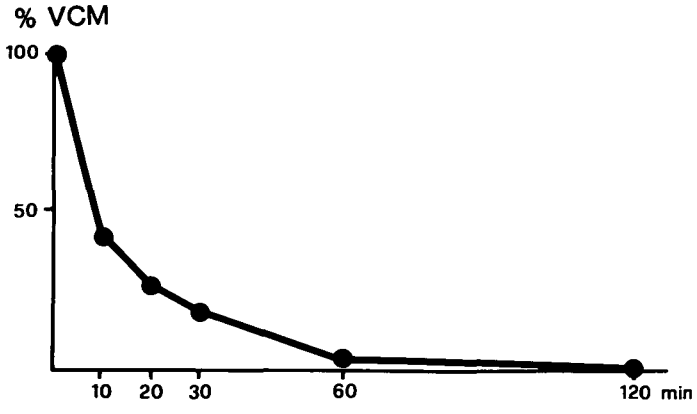

Fig. 2. Relative decrease of monomeric vinylchloride in the subcutaneous air pouch as a function of time after injection (100% = 2 mg/GP).

After this 6-day expression period, the cells were harvested and distributed into 12 dishes (10^5 cells/100 mm dish) containing the selective medium (15 μg 6-thioguanine/ml medium, 10% dialyzed FCS) and for the determination of the secondary cloning efficiency (II CE) three dishes (300 cells/100 mm dish) without the selective agent. After 8 days, clones were stained and counted: mutation frequencies were expressed as the number of mutants per 10^6 colony forming cells (Table I).

Results

Spontaneously arising mutations at the 6-thioguanine resistance locus in fibroblast like granulation tissue cells from animals only treated with the solvents were analysed: half of the control animals received 1 ml 2% ethanol in culture media, the others 1 ml mugolfen 620 injected into the air pouch. These controls were analysed concurrently with the test series. In Table I, control I represents concurrent control during the analyses of the first three chemicals and control II is the mean of the whole series.

After both direct and systemic administration, the control mutagen ENU induced mutation frequencies more than 100 greater than the spontaneous level. These increased mutation frequencies are comparable to those induced by other agents, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (32), procarbazine (33) and aristolochic acid (34) in the Granuloma Pouch Assay. Mutation frequencies determined after direct exposure of the target tissue to AFB_1, VCM and 2-AF, and after oral administration of 2-AAF (Table I; Figure 1), ranged from 5 to 10 times the spontaneous mutation frequency. The highest inducible mutation frequencies were 6–10% of those of the control mutagen.

No significant increase in mutagenic activity was detected after oral administration of AFB_1 (at levels of 0.23 and 0.93 mg/kg, one animal each, data not shown) or of 2-AF (Table I, Figure 1).

In the case of VCM, no clear dose–response relationship was established. Two possible reasons were further investigated. Firstly the presence over a given time interval of a non linear concentration of reactive monomers; secondly, that only a limited number of sensitive target cells is available in the tissue, for instance a (hypothetical) subpopulation in which phospholipid turnover can be specifically stimulated. VCM concentration was determined in samples taken from the air pouch at different time intervals after the initial VCM application (Figure 2). Mutation frequencies were determined in a subfraction of the freshly isolated cells separated by counterflow centrifugation (Beckman, J2-21 centrifuge, JE-6B elutriation system, Palo Alto, CA, USA; elutriation conditions: 4°C, Hanks balanced salt solution, 1500 r.p.m., 4.3–7.8 ml/min, theoretical size of 12–20 μm diameter) and representing 27.9% ± 3.6 (N = 4) of the total

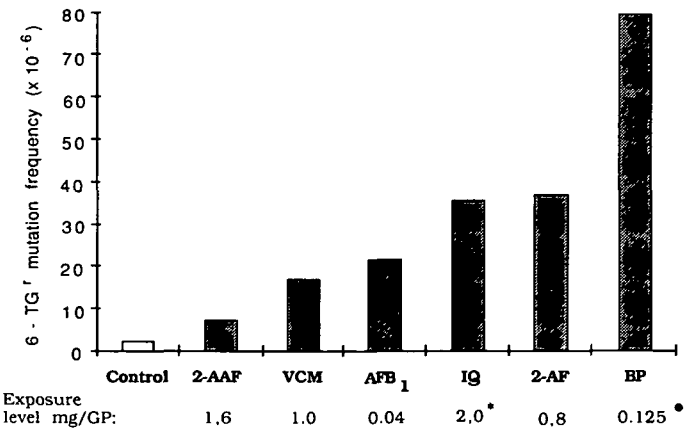

Fig. 3. Highest induced mutation frequencies at the 6-TG locus reported in the Granuloma Pouch Assay after single direct exposure of target cells. • Data from Maier *et al.*, 1980 and * Rademacher *et al.*, 1987.

cell population. Compared to the major fraction, the clone forming capacity of the separated cells was enhanced (Table I), their aldrin epoxidase activity increased by 30–90% (19) and the highest inducible mutation frequency doubled (Table I).

2-AAF was mutagenic only after oral administration: pretreatment of the animals (two i.p. administrations) increased the total number of mutants compared with p.o. administration alone (Table I, Figure 1).

Discussion

In the Granuloma Pouch Assay, the initial interaction of the test compound with DNA and the development of this primary lesion into a stable condition occur *in vivo*, whereas the expression of the mutant phenotype is facilitated by cell divisions *in vitro*: this restricts the analysis to cells with a high proliferative capacity, and therefore with a high probability of developing further into neoplastic cells.

At the HGPRT locus it is possible to detect base pair substitutions and small deletions which include the 6-TG^r-locus; as has been shown both after irradiation and exposure to a number of different chemicals (22,23). Functional, multilocus deletion mutants, however, cannot be detected efficiently (48,49); this is especially true for chemicals which initiate DNA-strand breaks. This might offer an explanation for the relatively low response, compared with the control mutagen of the chemicals tested.

Alternatively, these low mutation frequencies could be a consequence of a limited metabolic competence of the target tissue. The mutagenic activity detected after direct exposure of the granulation tissue suggests that pathways of cooxidation such as the prostaglandin synthetase (PHS) are involved. The following facts favour this interpretation: (i) the absence of cytochrome P-450-dependent mono-oxygenases in the granulation tissue (27), (ii) the oxidative conversion of aldrin to dieldrin mediated by the prostaglandin synthetase pathway (28) and by lipid peroxidation (29) in these target cells, (iii) the production of prostaglandin (PGE_2) by granulation tissue cells *ex vivo* (27), and (iv) the increased synthesis of prostaglandins and other hydroxy fatty acids which parallels tissue growth (9) during granuloma development in rats. However, the fact that prostaglandins influence cell proliferation (11) and DNA replication (40) in mammalian cells, and are involved in inflammatory processes in the rat air pouch (43) and in the growth of tumours (30), obstructed a direct investigation of the pathways involved in

metabolic activation. The use of specific inhibitors of the cyclo-oxygenase (e.g. indomethacin) will, therefore, also affect processes of mutation fixation. As a consequence, the modified mutation frequencies cannot be attributed unambiguously either to an initiation (mutation) or to a fixation (proliferation) step.

The mutagenic activity of AFB_1 after direct exposure was unexpected. However, the involvement of PHS metabolism in the mutagenicity of AFB_1 *in vitro* has been reported: DNA binding of AFB_1 is inhibited by indomethacin (2), and in bacterial tests AFB_1 is covalently bound as an epoxide to DNA when incubated with ram seminal vesicle, a source of PHS (6). *In vivo*, a low incidence of micronucleated polychromatophilic erythrocytes is found in extrahepatic tissue of rats in the micronuclei assay (46) after i.p. exposure to AFB_1, so, with this mode of administration, it is possible that unmetabolized AFB_1 could reach bone marrow cells. AFB_1 is also carcinogenic in extrahepatic tissues: it induces carcinomas at low incidence in the glandular stomach (10) and fibrosarcomas are inducible at the site of injection when AFB_1 is given several times in doses of 50 and 500 μg subcutaneously (12). However, the absence of mutagenic activity after oral application most likely is due to the efficient activation of AFB_1 in the liver, where the chemical is converted to the ultimate carcinogen, the reactive 2,3-oxide (20), in a single, cytochrome P-450-dependent step and interacts mainly at sites where these enzymes are accumulated. Accordingly, no reactive gene-mutation inducing metabolites are expected to reach the granulation tissue.

In contrast to AFB_1, 2-AAF and 2-AF are activated by an at least two step, cytochrome P-450-dependent mono-oxygenase process (22,51): in the case of 2-AAF this includes a deacetylation step (47). Both aromatic amines can be metabolized by ram seminal vesicles to species mutagenic in *in vitro* bacterial tests (45). The conversion of 2-AF to a gene-mutation inducing species in the Granuloma Pouch Assay agrees with these facts. *In vitro* 2-AF can be co-oxidized by the PHS-pathway (7) to products mutagenic in bacterial tests (8). The acetylated aromatic amine, however, can be less readily metabolized by peroxidative processes (38). A low deacetylase activity of the granulation tissue, in contrast to bladder tissue for instance (39), could explain the absence of mutagenic activity after direct exposure.

From data obtained after oral administration, we conclude that unlike 2-AF, 2-AAF may be converted into a fairly stable species by *N*-hydroxylation; thus reaching the granulation tissue. There, the hypothetical stable intermediate could be converted to the actual mutagen by *N,O*-acetyltransferases or sulphotransferases (1). The formation of this intermediate was increased when two intraperitoneal administrations preceeded the single administration by gavage (Figure 1): a stimulation of phase two conjugation reactions, or alternatively the overloading of detoxifying enzyme systems might be the reason. Using the micronucleus test in rats, 2-AAF dosed via oral gavage gave a weak positive response (4), but failed to induce a significantly increased number of micronuclei after i.p. application (52). It is possible that this mode of administration prevents the formation of a liver mediated, stable 2-AAF intermediate.

The involvement of cytochrome P-450-dependent mono-oxygenases in the metabolism of VCM (25,21) and in the formation of mutagenic metabolites in short term mutagenicity tests has been well documented (3,5,13). The mutagenic activity of VCM by direct exposure of the granulation tissue might be due to the reactive monomer *per se*: this molecule is lipophilic and probably membrane active. Thus, it could stimulate phospholipid turnover, and subsequently its own PHS mediated metabolic conversion. No data from other laboratories are available to support this hypothesis.

The non-linear dose-response relationship obtained with VCM can be explained by a non-proportional VCM concentration and a sensitive subpopulation. The observed rapid disappearance of the monomer probably due to rapid binding at the surrounding tissue or polymerization (Figure 2), might be further stimulated with increasing exposure levels. The concept of a small sensitive target population is supported by the enhanced mutation frequences found after exposure to VCM in a fraction of the freshly isolated granulation tissue cells showing enhanced aldrin epoxidase activity (Table I).

In conclusion, data obtained in the Granuloma Pouch Assay demonstrate that in extrahepatic tissues *in vivo* hepatocarcinogens can be converted to metabolites which are able to induce gene-mutations. In the light of the reported mutagenic activity also of benzo[*a*]pyrene (BP) (32) and 2-amino-3-methylimidazo[4,5-*f*]-quinoline (IQ) (44) in the Granuloma Pouch Assay, we propose that a co-oxidation pathway is involved, since both of these chemicals can be activated by prostaglandin H synthase-dependent pathways (BP: 37,50; IQ: 53). The highest reported induced mutation frequencies in the Granuloma Pouch Assay of these chemicals are summarized in Figure 3. Clearly, using the Granuloma Pouch Assay, the mutagenic potency of chemicals known to be activated by the PHS pathway *in vitro* or *ex vivo*, can be demonstrated *in vivo*.

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