Mutagenesis vol.15 no.1 pp.91–97, 2000

Aflatoxin B1-induced mitotic recombination in L5178Y mouse lymphoma cells

Verena Preisler, William J.Caspary[2,3], Florian Hoppe, Rudolf Hagen and Helga Stopper[1]

Department of Oto-Rhinolaryngology and [1]Department of Toxicology, University of Würzburg, Versbacher Straβe 9, 97078 Würzburg, Germany and [2]National Institutes of Health, Research Triangle Park, NC 27709, USA

Aflatoxin B1 is a human hepatocarcinogen. It is also a known point mutagen in bacteria and mammalian cells. This mutagenic activity may be at least partly responsible for its carcinogenic activity. However, recent studies show that aflatoxin B1 induces mitotic recombination in the yeast *Saccharomyces cerevisiae*. Because numerous reports have implicated mitotic recombination in mechanisms leading to carcinogenesis and because no one has shown that aflatoxin B1 induces recombination in mammalian cells, we decided to examine the ability of aflatoxin B1 to induce recombination in a mammalian cell line. We used a combination of methods, analysis for loss of heterozygosity and whole chromosome *in situ* hybridization, to identify mechanisms of chromosome mutation, including mitotic recombination in the mammalian L5178Y mouse lymphoma cell system. Our experiments revealed that mitotic recombination caused ~60% or more of the aflatoxin B1-induced mutagenic lesions in this cell system. Thus, mitotic recombination plays an important role in aflatoxin B1-induced mutagenesis in mammalian cells and possibly in chemically induced mutagenesis and carcinogenesis. This work suggests that multiple genetic lesions may be involved in aflatoxin B1-induced pathology.

Introduction

Aflatoxins are mycotoxins produced by a group of common fungal molds including *Aspergillus parasiticus* and *Aspergillus flavus*. These molds are ubiquitous in areas of the world with hot, humid climates. They are found in animal feed and contaminate human dietary staples in these climates (Sargeant *et al.*, 1961). Since countries in colder climates import food supplies from these regions, the potential toxic effects of aflatoxins are of concern world wide.

Aflatoxin B1 is a human hepatocarcinogen. It is also a liver carcinogen when fed to certain rodent species (Wogan and Newberne, 1967; Wogan *et al.*, 1973; IARC, 1993).

A case has been made that the mechanism leading to aflatoxin B1-induced cancer involves DNA adducts which are the precursors of guanosine to thymidine (G→T) transversions which are frequently observed in human liver (Garner *et al.*, 1988; Hsieh *et al.*, 1988; Lee *et al.*, 1989; Zhang *et al.*, 1991; Aguilar *et al.*, 1993; Eaton and Gallagher, 1994; Riley *et al.*, 1997). The following facts support this hypothesis: the urine of people exposed to aflatoxins contains aflatoxin B1–guanine adducts (Groopman *et al.*, 1992; Wild *et al.*, 1992) and most tumors from aflatoxin B1-contaminated regions harbor a G→T transversion at codon 249 in the *p53* gene (Bressac *et al.*, 1991; Hsu *et al.*, 1991; Murakami *et al.*, 1991; Scorsone, 1992 *et al.*; Li,D. *et al.*, 1993). It has also been shown that aflatoxin B1 induces point mutations in c-*ras* oncogenes although the role of these oncogenes in hepatocellular carcinoma has not been documented (McMahon *et al.*, 1986; Sinha *et al.*, 1988; Bauer Hofmann *et al.*, 1990; Soman and Wogan, 1993).

Sengstag *et al.* (1996) proposed that recombination might be involved in aflatoxin-induced carcinogenesis. Aflatoxin B1 strongly induced mitotic recombination, resulting in chromosome translocation and gene conversion events in a set of metabolically competent *Saccharomyces cerevisiae* yeast strains (Sengstag *et al.*, 1996; Sengstag, 1997). In contrast, this compound was only weakly mutagenic in a yeast strain sensitive to gene mutations. They concluded that metabolically activated aflatoxin B1 is much more prone to inducing mitotic recombination than gene mutation in yeast.

Although aflatoxin B1-induced recombinogenic activity has been shown to occur in yeast, its role in mammalian cell mutagenesis is not known. We decided to use L5178Y mouse lymphoma cells to examine the role of recombination in aflatoxin B1-induced mutagenesis (Clive *et al.*, 1972; Caspary *et al.*, 1997; Liechty *et al.*, 1998). These cells are heterozygous at the selectable thymidine kinase locus (*tk*) on chromosome 11 (Liechty *et al.*, 1993). If the active *tk* gene is inactivated or lost, the cells become resistant to the selective agent trifluorothymidine (TFT). This cell line detects a wide variety of mutagenic lesions, including point mutations, deletions and various types of chromosomal aberrations (Hozier *et al.*, 1992; Caspary *et al.*, 1997; Liechty *et al.*, 1998). The *in situ* protocol that we used captures essentially all the viable mutants, including the slowly growing mutants, for analysis. It also ensures the independence of the induced mutants (Rudd *et al.*, 1990).

To examine the possible role of mitotic recombination in aflatoxin B1 mammalian cell mutagenesis, we treated L5178Y mouse lymphoma cells with aflatoxin B1 and selected for mutants. We chose a dose that had the largest relative mutation fraction with little or no toxicity. We then isolated 41 mutant colonies, analyzed them for loss of heterozygosity (LOH) on chromosome 11 and, using fluorescent *in situ* hybridization, examined their metaphase spreads for their morphological characteristics. Our conclusion that mitotic recombination plays a role in aflatoxin B1-induced mutation shifts the emphasis of the mutational specificity of aflatoxin B1 away from point mutation toward large scale chromosome-type alterations.

Materials and methods

Cell culture

Mouse L5178Y cells, clone 3.7.2c (5), were cultured in suspension in RPMI-1640 supplemented with 95 U/ml penicillin, 95 µg/ml streptomycin, 0.25 mg/ml L-glutamine, 107 µg/ml sodium pyruvate and 10% heat-inactivated

[3]To whom correspondence should be addressed. Tel: +1 919 541 2150; Fax: +1 919 541 2242; Email: caspary@niehs.nih.gov

© UK Environmental Mutagen Society/Oxford University Press 2000

horse serum (Sigma Chemie GmbH, Deisenhofen, Germany). Cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37°C.

Mutation assay

We used the *in situ* procedure to obtain dose–response curves for mutation induction and for mutant isolation (Rudd *et al.*, 1990; Spencer and Caspary, 1994; Spencer *et al.*, 1994). Cultures of mouse L5178Y cells were treated with methotrexate before each experiment to kill pre-existing TFT-resistant (TFTr) cells. To accomplish this, cells were incubated for 24 h in culture medium plus methotrexate (0.3 µg/ml), thymidine (9 µg/ml), hypoxanthine (15 µg/ml) and glycine (22.5 µg/ml). The cells were then incubated for at least 24 h in the same medium without methotrexate. To measure chemically induced mutations using the *in situ* procedure, cultures containing 1 000 000 cells in 5 ml medium were treated. For metabolic activation, S9 mix (uninduced rat liver cell homogenate, supplemented with 1.5% citrate and 0.8% w/v NADPH) was added to the cells at a concentration of 100 µg protein/ml cell suspension.

Ethylmethanesulfonate (EMS) was used as a positive control without metabolic activation, and aminoacetylfluorene (AAF) was used (with S9 mix) as a positive control for metabolic activation. Two solvent controls (with and without S9 mix) containing ethanol (final concentration 1%) were used. We treated the cells with the chemicals for 3 h, then washed the cells twice with fresh medium. After that, 500 000 cells from each culture were added to 50 ml of semi-solid culture medium (containing 0.25% granulated agar; Baltimore Biological Laboratories), plated into two plastic 100 mm culture dishes and allowed to solidify at room temperature. TFTr cells were selected by adding an overlay of TFT to a final concentration of 8 µg/ml in 10 ml semi-solid medium after an expression time of 40 h. The cloning efficiency was determined by adding 600 cells to 100 ml of semi-solid medium and pouring into three plastic 100 mm dishes and allowed to solidify at room temperature. For picking mutants, two additional mutant plates were prepared as described above for 60 ng/ml aflatoxin B1. Mutants were picked from these two plates at day 7 after the start of the experiment under a microscope with a sterile pasteur pipette. Cells from each colony were dispersed into 1 ml of medium for culture and subcultured as required for a duration of 1–3 weeks. All other plates were incubated for a total of 9 (viability plates) or 12 days (mutant plates) at 37°C in 5% CO_2 for colony growth and the number of surviving colonies (viability plates) and TFTr colonies (mutant plates) were counted using an automatic colony counter.

Preparation of DNA for LOH analysis

DNA from 10 000 000 cells was isolated using a Qiagen kit (Qiagen, Hilden, Germany). Briefly, cells were lysed (AL buffer; Qiagen) and treated with proteinase K for 10 min at 70°C. DNA was precipitated with ethanol and purified using spin columns by washing twice with AW buffer (Qiagen). Elution of DNA was performed with AE buffer (Qiagen) at 70°C. DNA concentration and purity were determined photometrically at 260/280 nm.

LOH analysis

PCR reactions (20 µl) were prepared by mixing 10 µl of 2× PCR Master (Boehringer, Mannheim, Germany) containing 20 mM Tris–HCl, 100 mM KCl, 3 mM $MgCl_2$, 0.05 U/µl *Taq* DNA polymerase, 400 µM each dNTP and 0.01% Brij 35, pH 8.3, with 11.6 pmol of each primer, 3.5 µl of H_2O and 3 µl (30 ng) of template DNA. Reactions were performed in a Biozym PTC 100 thermal cycler (Hess, Oldendorf, Germany) by 'touchdown' PCR, in which the optimal annealing temperature was gradually approached from above to decrease the quantity of spurious PCR products resulting from some primer pairs. For primer Agl2, an initial 94°C denaturation for 2.5 min was followed by two cycles of denaturation at 94°C for 20 s, high temperature annealing at 72°C for 30 s and extension at 72°C for 20 s. The annealing temperature was decreased by 1°C for each additional two-cycle set until the final annealing temperature (66°C) was reached. Then 12 additional cycles were performed at 65°C, followed by extension at 72°C for 5 min. For all other primers, the procedure was essentially the same, except that the highest annealing temperature was 61°C, the lowest annealing temperature was 52°C and instead of the 12 cycles at 65°C, 20 cycles with an annealing temperature of 50°C were performed.

Gel electrophoresis

PCR products were analyzed on 10% non-denaturing polyacrylamide gels in 1× TBE buffer. Gel dimensions were 170 cm (w)×150 cm (h)×0.5 mm. Electrophoresis was performed for 4–6 h at 200 V and gels were stained with SybrGreen (15 µl/150 ml 1× TBE for 30 min) (Molecular Probes, OR) for visualization.

Preparation of metaphases

Cells from mutant colonies were subcultured daily for at least 5 days. Then colcemid (Gibco BRL, Eggenstein, Germany) was added at a concentration of 0.05 µg/ml. After 20 min the cell culture medium was replaced by hypotonic solution (0.075 M KCl at 37°C). After 15 min five drops of methanol/acetic acid (3:1) were added to the hypotonic solution to begin cell fixation. Then the cell suspension was centrifuged, carefully resuspended in methanol/acetic acid and incubated for 20 min at –20°C. This was repeated twice. Finally, cells were dropped onto glass slides and the slides were quickly air dried.

Table I. Aflatoxin B1-induced mutation in L5178Y mouse cells

Substance	Concentration	MnF	Rmnf	CE
Solvent	Ethanol (1%)	141		0.58
Solvent + S9	Ethanol (1%)	137	1	0.70
EMS	250 µg/ml	1273	9.3	0.44
AAF + S9	40 µg/ml	738	5.4	0.48
AFB1 + S9	5 ng/ml	214	1.6	0.70
AFB1 + S9	10 ng/ml	356	2.6	0.72
AFB1 + S9	20 ng/ml	430	3.1	0.53
AFB1 + S9	40 ng/ml	643	4.7	0.37
AFB1 + S9	60 ng/ml	909	6.6	0.55
AFB1 + S9	80 ng/ml	716	5.2	0.64
AFB1 + S9	100 ng/ml	1356	9.9	0.27

MnF, mutation fraction (no. of mutants per 1 000 000 viable cells); Rmnf, relative mutation fraction (mutant fraction relative to the control); CE, cloning efficiency (number of surviving colonies per plated cell); EMS, ethylmethanesulfonate; AAF, aminoacetylfluorene; AFB1, aflatoxin B1.

In situ hybridization

In situ hybridization was performed using a biotinylated mouse chromosome 11-specific paint (Caspary *et al.*, 1997; Liechty *et al.*, 1998). Slides were aged for 10–20 days. The painting probe was prepared by placing it in a thermocycler at 37°C for 5 min, 80°C for 5 min and 37°C for 2.5–3 h. The slides were denatured at 70°C for 75 s in formamide solution (70% in 2× SSC, pH 7.0). After dehydration in an ethanol series (70, 85, 90 and 100%, –20°C, 2 min each), slides were air dried. Next, slides were put on a slide warmer at 42°C and 10 µl of chromosome painting probe were added for overnight incubation in a humid chamber at 37°C. Slides were then washed for 3×5 min in formamide wash solution (55% in 2× SSC, pH 7.0) at 45°C, 3×5 min in 1× SSC at 45°C and then in 4× SSC at room temperature for at least 5 min. Detection and signal amplification were performed using materials and the protocol of Clontech Laboratories (Palo Alto, CA) for FITC detection of biotinylated DNA. Counterstaining of whole DNA was achieved with bisbenzimide 33258 (5 µg/ml, 2.5 min). In the final step, a second counterstaining of whole DNA was achieved by adding mounting medium containing 0.3 µg/ml propidium iodide. We limited the analysis to mutants that showed LOH for at least 25% of the total chromosome 11 length because mutants with smaller losses would be difficult to differentiate from mutants with normal chromosomes using FISH. Metaphases from each mutant colony were analyzed for the number of chromosome 11 homologs and for chromosome 11 centromere sizes. The centromere size was also analyzed using bizbenzimide stain, which shows the C band regions equivalent to the centromere. The lengths of chromosomes 11 were visualized under the microscope. We required 10 good metaphases showing consistent results before drawing conclusions.

Results

Aflatoxin B1-induced mutations in the L5178Y mouse lymphoma mutation assay at the tk locus

The mutation fraction was dose dependent (Table I). We prepared replicate plates for all the doses and chose a concentration of 60 ng/ml for mutant isolation because of its high relative mutation fraction and high cloning efficiency (no or little toxicity). The relative mutation fraction of 6.6 for this concentration implies that 84% of the mutant colonies are aflatoxin B1 induced (Table I).

Loss of heterozygosity

To determine the types of lesions present in these mutants, we examined them for LOH. We isolated 41 aflatoxin B1-treated mutant clones. The *in situ* protocol that we used to obtain and isolate the mutants ensured that the induced mutant colonies were independent (Rudd *et al.*, 1990). We PCR amplified up

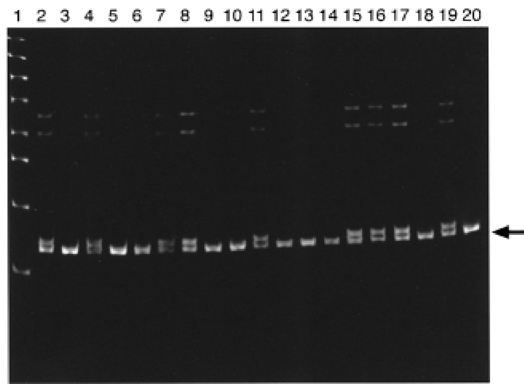

Fig. 1. LOH analysis by gel electrophoresis using primer Mit59. Lane 1 contains a 123 bp DNA ladder, lane 19 shows heterozygous (wild-type) L5178Y cells, lane 20 shows a mutant with known LOH (positive control) and lanes 2–18 several aflatoxin B1-induced mutants (nos 3, 11, 12, 13, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 30 and 56). An arrow indicates the bands that were analyzed for LOH detection.

to six heteromorphic microsatellite sites along chromosome 11 in each of these clones. These loci were from 2 (Mit2) to 78 cM (Agl2) from the centromere (Figure 1). The total length of chromosome 11 is ~80 cM. The most distal probe was Agl2, which resides at a heteromorphic site within the *tk* gene between exons 6 and 7 (Liechty *et al.*, 1996b). Figure 2 shows an example of our results using the probe Mit59.

We categorized the mutants as group 1, 2 or 3 mutants. Into group 1 we placed 10 of the 41 mutants (24%) showing no LOH. Group 2 comprised 26 mutants (63%) showing LOH at some of the loci. Group 3 was composed of five mutants (12%) showing LOH at all analyzed sites (Figure 2 and Table II). In all mutants showing LOH, the losses included Agl2, which resides within the *tk* gene.

The LOH data suggests that all the group 2 and 3 mutants possessed chromosomal lesions. The 10 mutants from group 1 showed no LOH with any of the probes. These mutants were potential point mutants. However, some of them might have small chromosomal lesions encompassing sequences distal to Agl2 down to the distal end of the chromosome or sequences proximal to Agl2 and distal to Mit103.

In situ hybridization

A limitation of the LOH analysis is that it is not always possible to identify the mechanisms leading to LOH. Specifically, translocations and changes in chromosome number are not detectable and mitotic recombination is not distinguishable from deletion. For example, if LOH occurs at all the loci we examined, mitotic recombination that includes those loci is not distinguishable from chromosome loss. To overcome these shortcomings, we combined the results from LOH analysis with those from whole chromosome 11 *in situ* hybridization. This technique allows ready identification of chromosome 11 sequences in these cells (Liechty *et al.*, 1996a; Caspary *et al.*, 1997).

The sizes of the two centromeres from the homologous chromosomes are different. This difference has been used to distinguish the two homologous chromosomes (Hozier *et al.*, 1982; Sawyer *et al.*, 1985; Blazak *et al.*, 1986). The homolog with the larger centromere contains the functional *tk* allele (Hozier *et al.*, 1982). We used this difference to identify the origin of the centromeric regions of these two homologous chromosomes cytogenetically (e.g. bisbenzimide stain) and by FISH. All conclusions about centromere size were based

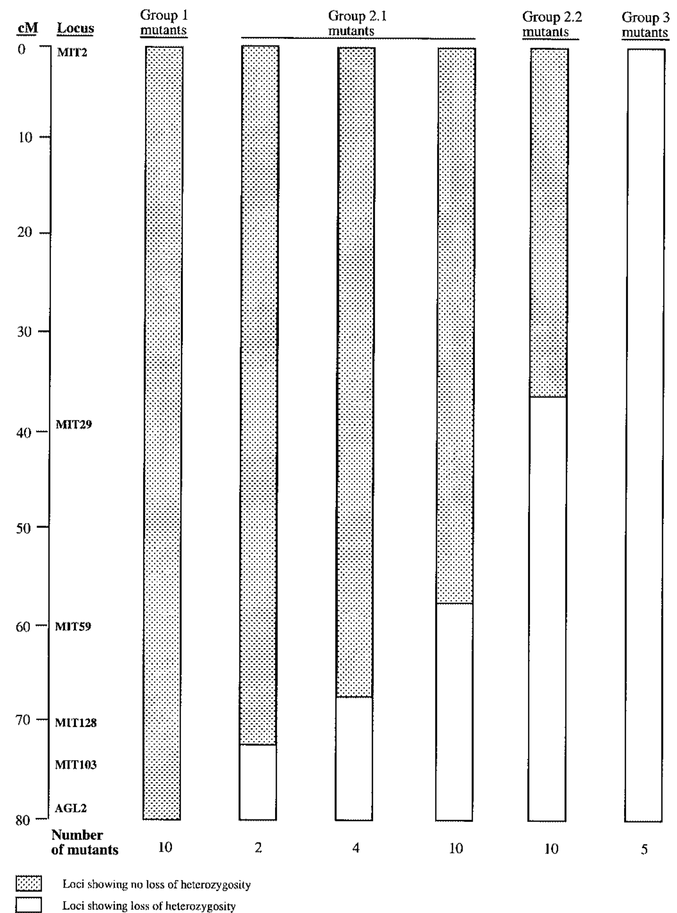

Fig. 2. LOH of aflatoxin-induced mutants. The six markers are listed on the vertical axis at locations showing their distances in cM from the centromere. The mutants are listed by group number. The 10 group 1 mutants showed no LOH as indicated by the speckled bar. Group 2 mutants showed partial LOH. For example, two mutants showed losses at Agl2 and Mit103, but no losses at the other markers. Group 3 mutants showed LOH at all the markers examined.

Table II. LOH results of 41 aflatoxin B1-induced L5178Y mouse lymphoma mutants using six informative microsatellites located on chromosome 11

Observation	Group	Total mutants	Percentage
No LOH at any locus examined	1	10	24
LOH at some loci examined	2	26	64
LOH at all loci examined	3	5	12
Total		41	100

One of the microsatellites, Agl2, is located within the *tk1* gene. The remainder were all proximal to *tk1*. Not all microsatellite loci were analyzed for group 1 mutants. Since Agl2 and Mit103 were present in all these mutants, the mutation could not extend to regions closer to the centromere than Mit103.

on the examination of at least 10 good metaphases from each mutant.

We limited FISH analysis to mutants that showed LOH for at least 25% of the total chromosome 11 length, i.e. mutants that show loss from Agl2 (located at 81 cM) to Mit59 (located at 61 cM). Mutants with smaller losses would be difficult to differentiate from mutants with normal chromosomes using FISH. Fifteen mutants fulfilled this requirement: 10 from group 2.2 and all five from group 3 (see Figure 3 for an example).

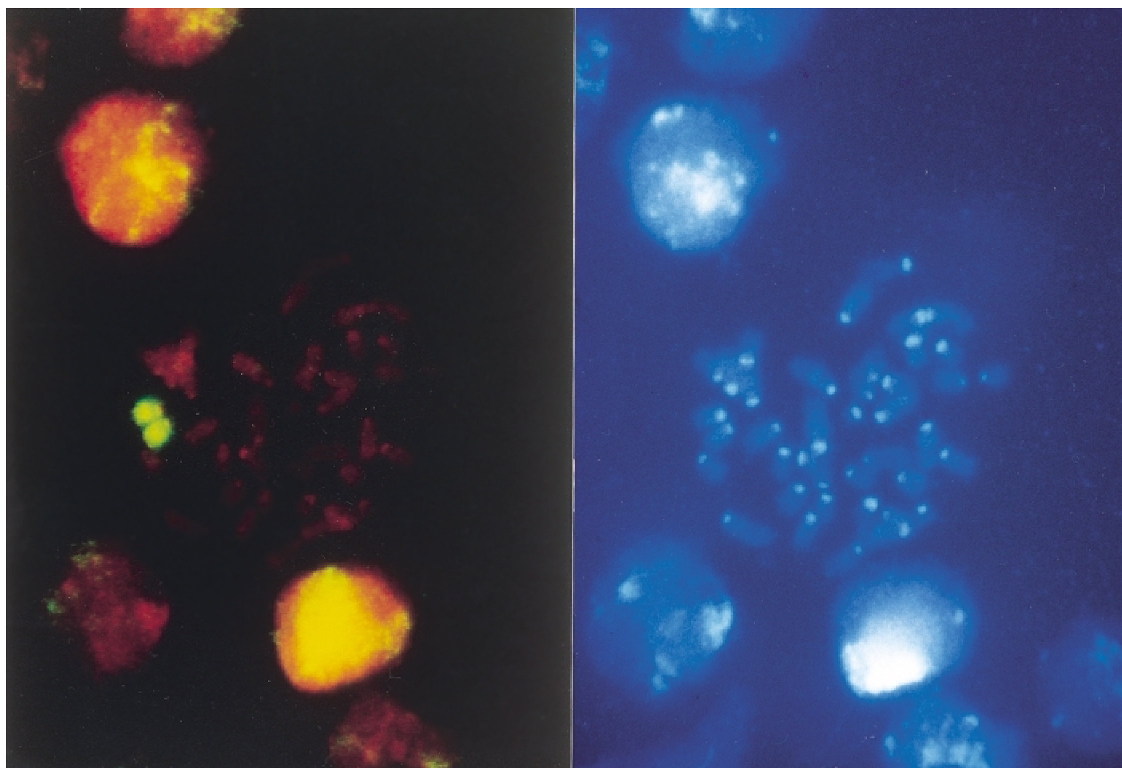

Fig. 3. Metaphase of an aflatoxin B1-induced L5178Y mouse cell mutant colony. *In situ* hybridization using chromosome 11 painting probe shows two chromosome 11 alleles of equal lengths (yellow stain, left; DNA counterstain propidium iodide) with unequal centromere sizes (bisbenzimide stain, right). This mutant shows LOH of only part of chromosome 11 and must therefore be due to mitotic recombination.

We did not analyze any of the group 1 mutants by *in situ* hybridization because these lesions were either point mutations or small intragenic or intergenic lesions. Although mutants in this class that were due to intragenic or intergenic events could result from small recombinations, they would look normal by *in situ* hybridization because of the small size of the losses.

All five mutants from group 3 (mutants 6, 18, 52, 56 and 57) were analyzed by *in situ* hybridization (Table III). Three of these mutants had two chromosomes 11 of apparently equal length and different centromere sizes. Since the two original centromeres were present, recombination was responsible for the TFT resistance expressed by these mutants. The two remaining mutants showed LOH at all tested sites. As shown in Table III, these mutants were monosomic for chromosome 11. Therefore, analysis of centromere size and chromosome lengths was not possible. Ten mutants in group 2 had damage large enough to have interpretable data after chromosome painting (Table III). Four of these (25, 13, 21 and 58) were trisomic for chromosome 11. Because not all the sites showed LOH, we suggest that recombination was the primary event causing the mutation. It appears that in all four cases, a secondary event was the duplication of chromosome 11 containing the tk^- allele since the centromere size of two of the chromosomes was small.

In five of these mutants (9, 11, 24, 26 and 22), two chromosomes 11 were present and had different centromere sizes. The chromosome 11 with the large centromere was elongated (~30–50% additional length). Since not all the sites showed LOH, recombination must have been the mechanism leading to these mutations. One mutant in this group was monosomic for chromosome 11. Therefore, analysis of centromere size and chromosome lengths for this mutant was not possible.

Discussion

Interest in the expression of recessive genes is due to the importance of tumor suppressor genes in carcinogenesis (Müller and Scott, 1992). Loss of the second allele of a tumor suppressor gene in a cell that has already sustained a mutation (often a point mutation) in the first allele is a critical step in carcinogenesis. Tumor suppressor genes require the loss of function of both of the homologous alleles to express their phenotypes and it is believed that the second step is often a recombination event (Cavenee *et al.*, 1983).

A powerful approach to assessing the role of recombination in mammalian cells is the use of LOH analysis with fluorescent *in situ* hybridization. Recent reports demonstrated the successful use of these techniques in mouse lymphoma cells (Caspary *et al.*, 1997; Liechty *et al.*, 1998).

There are many reports showing that aflatoxin B1 causes point mutations (Swenson *et al.*, 1977; Aguila *et al.*, 1993; Gerbes and Caselmann, 1993; Eaton and Gallagher, 1994; Bailey *et al.*, 1996; Choi *et al.*, 1996). The analysis to be presented here shows that aflatoxin B1 also causes the expression of mutations mediated by recombinogenic events in mammalian cells. This extends previous reports that aflatoxin B1 causes recombination in yeast (Sengstag and Wurgler, 1994; Sengstag *et al.*, 1996) and lends credence to the idea that aflatoxin B1-induced chromosomal mutations manifested by LOH may lead to hepatocarcinogenesis.

Our analysis shows that the majority of aflatoxin B1-treated mutants were recombinants. In this analysis we exclude the

Table III. *In situ* hybridization of aflatoxin B1-induced mutants using a chromosome 11 paint

Mutant label	No. of chr. 11	Length of chr. 11	Centromere size	LOH	Interpretation	Remarks
Group 2 mutants						
25	3	Equal	One large, two small[a]	Agl2→Mit29	Recombination and doubling of chr. 11–	
13	3	One elongated[b]	One large (long allele), two small[a]	Agl2→Mit59	Recombination (unequal reciprocal exchange) and doubling of chr. 11–	*In situ* signal of the long chr. is stronger; reason unknown
21	3	One elongated[b]	One large (long allele), two small[a]	Agl2→Mit59	Recombination (unequal reciprocal exchange) and doubling of chr. 11–	
58	3	One elongated[b]	One large (long allele), two small[b]	Agl2→Mit29	Recombination (unequal reciprocal exchange) and doubling of chr. 11–	Some metaphases are polyploid (~20%)
9	2	Equal	Large and small	Agl2→Mit29	Recombination	
11	2	Equal	Large and small	Agl2→Mit29	Recombination	
24	2	Equal	Large and small	Agl2→Mit59	Recombination	
26	2	Equal	Large and small	Agl2→Mit59	Recombination	
22	2	Slightly different	Large (longer chr.) and small	Agl2→Mit29	Recombination	Possibly unequal exchange
4	1	nd[c]	nd[c]	Agl2→Mit59[c]	Recombination, then loss of one chr. 11[d]	No translocation of chr. 11 sequences to other chromosomes were visible
Group 3 mutants						
18	2	Equal	Different	All sites	Recombination	
52	2	Equal	Different	All sites	Recombination	
57	2	Equal	Different	All sites	Recombination	
6	1	nd[c]	nd[c]	All sites	Probably loss of chr. 11 harboring the tk^+ allele	No translocation of chr. 11 fragments to other chr. were visible
56	1	nd[c]	nd[c]	All sites	Probably loss of chr. 11 harboring the tk^+ allele	No translocation of chr. 11 fragments to other chr. were visible

The classification groups 2 and 3 were defined in Table II. The mutants shown in this table were those examined by *in situ* hybridization. Chr., chromosome; chr. 11–, chromosome 11 harboring the tk^- allele.

[a]Centromere sizes of the smaller chromosomes may be different. If so, the three chromosomes would have three centromere sizes. Were this the case, recombination would still explain the two chromosomes with the smaller and larger centromere. The explanation for the third centromere size is not readily apparent.

[b]The large chromosome is ~20–50% larger than the two small chromosomes.

[c]nd, not determinable because there is no internal standard (the homologous chromosome is not present). However, an examination of many metaphases suggests that the chromosome is longer than normal and that the centromere is large. If so, the lost chromosome must be the original chromosome containing the tk^- allele.

[d]Because LOH only extnds to Mit59 (no LOH at Mit2), the primary event causing mutation must be recombination. The secondary event is then the loss of chromosome 11–.

three monosomic mutants because the mechanism of their formation is not known. Exclusion of these three mutants will not qualitatively affect our conclusions. Our sampling of the mutants from groups 2 and 3 indicated that the 12 mutants from groups 2.2 and 3 resulted from recombination. We would expect that the remaining mutants from group 2 (those with LOH too small to be analyzed by *in situ* hybridization) were also due to recombination. This assumption is justified because the only difference between group 2.1 and 2.2 (Figure 2) mutants was the size of the LOH observed. There is no evidence that the mechanisms of formation are different. Of the 41 mutants isolated, it appears that at least 28 were due to recombination. This number could be higher if some of the 10 mutants from group 1 were not point mutations but small recombinogenic lesions.

To estimate the percentage of aflatoxin B1-induced mutants that were recombinants, we have to consider the effect of spontaneous mutants. The relative mutation fraction of 6.6 tells us that 16% of the 41 isolated mutants in this study may be of spontaneous origin. Previous studies have shown that ~70% of spontaneous mutants contain recombinations with the remainder harboring gene mutations or small partially intragenic deletions (Liechty *et al.*, 1996a, 1998; Caspary *et al.*, 1997). Thus, of the 41 mutants examined here, seven (16%) could have been of spontaneous origin, of which approximately five (70%) could have harbored recombinogenic events. Thus, of the 28 mutants in this study harboring recombinations, aflatoxin B1 induced at least 23. Since 34 mutants were induced (41–7), 68% (23 of 34) of the aflatoxin B1-induced mutants were due to recombination. Even if all seven spontaneous mutants in our investigation were recombinants, 21 of the recombinants were aflatoxin B1 induced and, therefore, 62% (21 of 34) of the induced mutants were due to recombination. Thus, the majority of aflatoxin B1-induced mutants were recombinants.

The high recombinogenic activity of aflatoxin B1 is in agreement with the findings of Sengstag and co-workers (Senstag and Wurgler, 1994; Sengstag *et al.*, 1996; Sengstag, 1997) in yeast and those of Zhang *et al.* (1991), who used a mammalian system for intrachromosomal recombination. Removal of an inserted sequence by recombination reactivates the *HPRT* gene in a V79 Chinese hamster cell line (SP5) and is detectable using a reversion mutation assay. They observed statistically significant enhancements in the frequency of reversion in SP5 cells after treatment with aflatoxin B1.

These data extend the list of chemicals that are known to induce mitotic recombination in mammalian cells (Hozier *et al.*, 1992; Li,C.Y. *et al.*, 1992; Smith and Grosovsky, 1993; Zhu *et al.*, 1993; Xia *et al.*, 1994). It also shows that mitotic recombination plays an important role in aflatoxin B1-induced mutagenesis in mammalian cells and possibly in chemically induced mutagenesis and carcinogenesis.

Acknowledgement

We thank Mrs H. Raabe for her expert technical assistance. We thank Professor Wolfgang Dekant and Dr Jeff French for their comments on this manuscript. This work was supported by a NATO CRG program grant (no. 940684) to W.J.C. and H.S. and by SFB 172 of the Deutsche Forschungsgemeinschaft to H.S.

References

Aguilar,F., Hussain,S.P. and Cerutti,P. (1993) Aflatoxin B1 induces the transversion of G→T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proc. Natl Acad. Sci. USA*, **90**, 8586–8590.

Bailey,E.A., Iyer,R.S., Stone,M.P., Harris,T.M. and Essigmann,J.M. (1996) Mutational properties of the primary aflatoxin B1–DNA adduct. *Proc. Natl Acad. Sci. USA*, **93**, 1535–1539.

Bauer Hofmann,R., Buchmann,A., Wright,A.S. and Schwarz,M. (1990) Mutations in the Ha-*ras* proto-oncogene in spontaneous and chemically induced liver tumours of the CF1 mouse. *Carcinogenesis*, **11**, 1875–1877.

Blazak,W., Stewart,B., Galperin,I., Allen,K., Rudd,C., Mitchell,A. and Caspary,W. (1986) Chromosome analysis of trifluorothymidine-resistant L5178Y mouse lymphoma cell colonies. *Environ. Mutagen.*, **8**, 229–240.

Bressac,B., Kew,M., Wands,J. and Ozturk,M. (1991) Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa [see comments]. *Nature*, **350**, 429–431.

Caspary,W.J., Stopper,H., Hozier,J., Liechty,M. and Davis,L. (1997) Mechanisms leading to the expression of recessive alleles: the use of polymorphic microsatellites and whole chromosome painting probes to analyze mouse tumors, mutants and micronuclei. In Mueller-Hermelink,H.K. (ed.), *Risk and Progression Factors in Carcinogenesis*. Springer-Verlag, Berlin, Vol. 143, pp. 161–182.

Cavenee,W.K., Dryja,T.P., Phillips,R.A., Benedict,W.F., Godbout,R., Gallie,B.L., Murphree,A.L., Strong,L.C. and White,R.L. (1983) Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature*, **305**, 779–784.

Choi,M.J., Lee,J.W. and Lee,B.M. (1996) Comparative assessment of DNA adduct formation, *Salmonella* mutagenicity and chromosome aberration assays as short-term tests for DNA damage. *J. Toxicol. Environ. Health*, **49**, 271–284.

Clive,D., Flamm,W.G., Machesko,M.R. and Bernheim,N.J. (1972) Mutational assay system using the thymidine kinase locus in mouse lymphoma cells. *Mutat. Res.*, **16**, 77–87.

Eaton,D.L. and Gallagher,E.P. (1994) Mechanisms of aflatoxin carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.*, **34**, 135–172.

Garner,R.C., Dvorackova,I. and Tursi,F. (1988) Immunoassay procedures to detect exposure to aflatoxin B1 and benzo(*a*)pyrene in animals and man at the DNA level. *Int. Arch. Occup Environ. Health*, **60**, 145–150.

Gerbes,A.L. and Caselmann,W.H. (1993) Point mutations of the P53 gene, human hepatocellular carcinoma and aflatoxins. *J. Hepatol.*, **19**, 312–315.

Groopman,J.D., Zhu,J.Q., Donahue,P.R., Pikul,A., Zhang,L.S., Chen,J.S. and Wogan,G.N. (1992). Molecular dosimetry of urinary aflatoxin–DNA adducts in people living in Guangxi Autonomous Region, People's Republic of China. *Cancer Res.*, **52**, 45–52.

Hozier,J., Sawyer,J., Clive,D. and Moore,M. (1982) Cytogenetic distinction between the TK^{+} and TK^{-} chromosomes in the L5178Y $TK^{+/-}$ 3.7.2C mouse-lymphoma cell line. *Mutat. Res.*, **105**, 451–456.

Hozier,J., Applegate,M. and Moore,M.M. (1992) *In vitro* mammalian mutagenesis as a model for genetic lesions in human cancer. *Mutat. Res.*, **270**, 201–209.

Hsieh,L.L., Hsu,S.W., Chen,D.S. and Santella,R.M. (1988) Immunological detection of aflatoxin B1–DNA adducts formed *in vivo*. *Cancer Res.*, **48**, 6328–6331.

Hsu,I.C., Metcalf,R.A., Sun,T., Welsh,J.A., Wang,N.J. and Harris,C.C. (1991) Mutational hotspot in the p53 gene in human hepatocellular carcinomas [see comments]. *Nature*, **350**, 427–428.

IARC (1993) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins*. IARC Scientific Publications no. 56, IARC, Lyon, pp. 245–396.

Lee,H.S., Sarosi,I. and Vyas,G.N. (1989) Aflatoxin B1 formamidopyrimidine adducts in human hepatocarcinogenesis: a preliminary report. *Gastroenterology*, **97**, 1281–1287.

Li,C.Y., Yandell,D.W. and Little,J.B. (1992) Molecular mechanisms of spontaneous and induced loss of heterozygosity in human cells *in vitro*. *Somat. Cell Mol. Genet.*, **18**, 77–87.

Li,D., Cao,Y., He,L., Wang,N.J. and Gu,J.R. (1993) Aberrations of *p53* gene in human hepatocellular carcinoma from China. *Carcinogenesis*, **14**, 169–173.

Liechty,M.C., Rauchfuss,H.S., Lugo,M.H. and Hozier,J.C. (1993) Sequence analysis of tk_a^{-}-1 and tk_b^{+}-1 alleles in L5178Y $tk^{+/-}$ mouse-lymphoma cells and spontaneous $tk^{-/-}$ mutants. *Mutat. Res.*, **286**, 299–307.

Liechty,M., Scalzi,J., Davis,L., Spencer,D., Hozier,J. and Caspary,W. (1996a) Recombination and terminal deletions in L5178Y mouse cells. *Environ. Mol. Mutagen.*, **27** (suppl. 27), 41.

Liechty,M.C., Crosby,H., Murthy,A., Davis,L.M., Caspary,W.J. and Hozier,J.C. (1996b) Identification of a polymorphic microsatellite within the *tk* gene in L5178Y mouse lymphoma cells. *Mutat. Res.*, **371**, 265–271.

Liechty,M., Scalzi,J., Sims,K., Crosby,H., Spencer,D., Davis,L., Caspary,W. and Hozier,J. (1998) Analysis of large and small colony L5178Y *ti–/–* mouse lymphoma mutants by loss of heterozygosity (LOH) and by whole

chromosome 11 painting: detection of recombination. *Mutagenesis*, **13**, 461–474.

Müller,H. and Scott,R. (1992) Hereditary conditions in which the loss of heterozygosity may be important. *Mutat. Res.*, **284**, 15–24.

McMahon,G., Hanson,L., Lee,J.J. and Wogan,G.N. (1986) Identification of an activated c-Ki-ras oncogene in rat liver tumors induced by aflatoxin B1. *Proc. Natl Acad. Sci. USA*, **83**, 9418–9422.

Murakami,Y., Hayashi,K., Hirohashi,S. and Sekiya,T. (1991) Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. *Cancer Res.*, **51**, 5520–5525.

Riley,J., Mandel,H.G., Sinha,S., Judah,D.J. and Neal,G.E. (1997) *In vitro* activation of the human Harvey-*ras* proto-oncogene by aflatoxin B1. *Carcinogenesis*, **18**, 905–910.

Rudd,C., Daston,D. and Caspary,W. (1990) Spontaneous mutation rates in mammalian cells: effect of differential growth rates and phenotypic lag. *Genetics*, **126**, 435–442.

Sargeant,K., O'Kelly,J., Carnaghan,R. and Allcroft,R. (1961) The assay of a toxic principle in certain groundnut meals. *Vet. Rec.*, **73**, 1219–1222.

Sawyer,J., Moore,M.M., Clive,D. and Hozier,J. (1985) Cytogenetic characterization of the L5178Y $TK^{+/-}$ 3.7.2C mouse lymphoma cell line. *Mutat. Res.*, **147**, 243–253.

Scorsone,K.A., Zhou,Y.Z., Butel,J.S. and Slagle,B.L. (1992) p53 mutations cluster at codon 249 in hepatitis B virus-positive hepatocellular carcinomas from China. *Cancer Res.*, **52**, 1635–1638.

Sengstag,C. (1997) The molecular mechanism of aflatoxin B1-induced liver cancer: is mitotic recombination involved? *Mol. Carcinog.*, **19**, 147–152.

Sengstag,C. and Wurgler,F.E. (1994) DNA recombination induced by aflatoxin B1 activated by cytochrome P450 1A enzymes. *Mol. Carcinog.*, **11**, 227–235.

Sengstag,C., Weibel,B. and Fasullo,M. (1996) Genotoxicity of aflatoxin B1: evidence for a recombination-mediated mechanism in *Saccharomyces cerevisiae*. *Cancer Res.*, **56**, 5457–5465.

Sinha,S., Webber,C., Marshall,C.J., Knowles,M.A., Proctor,A., Barrass,N.C. and Neal,G.E. (1988) Activation of ras oncogene in aflatoxin-induced rat liver carcinogenesis. *Proc. Natl Acad. Sci. USA*, **85**, 3673–3677.

Smith,L.E. and Grosovsky,A.J. (1993) Genetic instability on chromosome 16 in a human B lymphoblastoid cell line. *Somat. Cell Mol. Genet.*, **19**, 515–527.

Soman,N.R. and Wogan,G.N. (1993) Activation of the c-Ki-ras oncogene in aflatoxin B1-induced hepatocellular carcinoma and adenoma in the rat: detection by denaturing gradient gel electrophoresis. *Proc. Natl Acad. Sci. USA*, **90**, 2045–2049.

Spencer,D.L. and Caspary,W.J. (1994) *In situ* and suspension protocols for mutation in L5178Y mouse cells: dose response and colony size distribution. *Mutat. Res.*, **322**, 291–300.

Spencer,D.L., Hines,K.C. and Caspary,W.J. (1994) An *in situ* protocol for measuring the expression of chemically-induced mutations in mammalian cells. *Mutat. Res.*, **312**, 85–98.

Swenson,D., Lin,J., Miller,E. and Miller,J. (1977) Aflatoxin B_1-2,3-oxide as a probable intermediate in the covalent binding of aflatoxins B_1 and B_2 to rat liver DNA and ribosomal RNA *in vivo*. *Cancer Res.*, **37**, 172–181.

Wild,C.P., Hudson,G.J., Sabbioni,G., Chapot,B., Hall,A.J., Wogan,G.N., Whittle,H., Montesano,R. and Groopman,J.D. (1992) Dietary intake of aflatoxins and the level of albumin-bound aflatoxin in peripheral blood in The Gambia, West Africa. *Cancer Epidemiol. Biomarkers Prev.*, **1**, 229–234.

Wogan,G. (1973) Aflatoxin carcinogenesis. In Busch,H. (ed.), *Methods in Cancer Research*. Academic Press, New York, NY, pp. 309–344.

Wogan,G.N. and Newberne,P.M. (1967) Dose–response characteristics of aflatoxin B_1 carcinogenesis in the rat. *Cancer Res.*, **27**, 2370–2376.

Xia,F., Amundson,S.A., Nickoloff,J.A. and Liber,H.L. (1994) Different capacities for recombination in closely related human lymphoblastoid cell lines with different mutational responses to X-irradiation. *Mol. Cell. Biol.*, **14**, 5850–5857.

Zhang,Y.J., Chen,C.J., Lee,C.S., Haghighi,B., Yang,G.Y., Wang,L.W., Feitelson,M. and Santella,R. (1991) Aflatoxin B1–DNA adducts and hepatitis B virus antigens in hepatocellular carcinoma and non-tumorous liver tissue. *Carcinogenesis*, **12**, 2247–2252.

Zhu,Y., Stambrook,P.J. and Tischfield,J.A. (1993) Loss of heterozygosity: the most frequent cause of recessive phenotype expression at the heterozygous human adenine phosphoribosyltransferase locus. *Mol. Carcinog.*, **8**, 138–144.

Received on August 9, 1999; accepted on September 24, 1999