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Chrysotile asbestos fibers mediate homologous recombination in Rat2λ fibroblasts: implications for carcinogenesis

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Abstract

Asbestos fibers are widespread environmental carcinogens whose mutagenicity is now established. Nonetheless, the molecular nature of these mutations and the mechanisms by which they accelerate carcinogenesis remain poorly understood. We have assessed the ability of asbestos fibers to promote homologous recombination, a potent mechanism for generating intrachromosomal rearrangements, such as deletions, and mitotic recombination. For this, we have developed a new assay which determines the extent to which a marker gene present in DNA introduced by asbestos can recombine with homologous genes residing in a transfected cell. We have demonstrated that Calidria chrysotile fibers are mutagenic and are able to mediate transfection of molecularly marked mutant *lacI* genes in a manner that results in their preferential recombination with homologous wild-type genes in the transfected cell. Asbestos induced recombination events may play a significant role in asbestos mutagenesis and carcinogenesis, and promotion of recombination may underlie the well-recognized synergy of asbestos with other carcinogens.

Keywords: Chrysotile asbestos; *lacI*; Homologous recombination; Rat2λ cell

1. Introduction

Several different types of asbestos fibers are carcinogenic in humans (Selikoff et al., 1964; Nicholson, 1986; Begin et al., 1992) and experimental animals (Wagner et al., 1974). Asbestos fibers can damage DNA (Libbus et al., 1989; Kamp et al., 1992, 1995), cause mutations (Fasy, 1991; Hei, 1991;

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Hei et al., 1992; Stankowski et al., 1992), induce aneuploidy (Jaurand, 1989; Walker et al., 1992) and mediate transfection of exogenous DNA (Appel et al., 1988; Gan et al., 1993). In addition, asbestos fibers have been proposed to act as tumor promoters (Selikoff et al., 1968; Jaurand, 1989; Fasy, 1991; Walker et al., 1992). The relative contributions of these different mechanisms to asbestos carcinogenesis remain undefined. Although asbestos-mediated mutagenesis has been confirmed in several different cell culture assays (Fasy, 1991; Hei, 1991; Hei et al., 1992; Stankowski et al., 1992), the molecular mechanisms have yet to be characterized. Moreover, the ability of asbestos fibers to synergistically enhance the effect of other carcinogens and mutagens (Selikoff et al., 1968; Jaurand, 1989; Fasy, 1991; Hei, 1991; Walker et al., 1992), although well recognized and frequently studied, has for almost three decades, remained unexplained in molecular terms.

Homologous recombination is one mechanism which might readily contribute to both the mutagenicity and carcinogenicity of asbestos fibers. All types of asbestos fibers have been shown to mediate cell transfection (Appel et al., 1988; Gan et al., 1993) and transfection is frequently employed in the laboratory to generate homologous recombination (Waldman, 1992; Savransky et al., 1994). Homologous recombination can give rise to deletions and genomic rearrangements (Schiestl, 1989). Cells which have already sustained an inactivating mutation in one allele of a tumor suppressor gene are particularly vulnerable to the procarcinogenic potential of mitotic recombination (Passarge and Bartram, 1976; Cavenee et al., 1983; Knudson, 1986) which arises not from residual sequence changes at the point of recombination, but from the potential maldistribution of mutant tumor suppressor alleles. In light of these considerations, it is important to assess the capacity of asbestos fibers to modulate the frequency of homologous recombination events. Following asbestos-mediated transfection of DNA, the introduced DNA is damaged and fragmented upon entry into the host cell (Appel et al., 1988; Gan et al., 1993). Such DNA fragments could be mutagenic by recombining with homologous sequences in the genome. Here we have employed a new rat cell mutagenesis assay which demonstrates that chrysotile asbestos promotes a high level of homologous recombination between exogenous plasmid DNA and specific genes within the host cell genome.

2. Materials and methods

2.1. Detection of mutations in the lacI gene

Exposures for mutagenicity testing were done as follows. Rat2λ cells (Wyborski et al., 1995) were obtained from Stratagene and grown at 37°C and 5% CO_2 in 75 cm^2 flasks (Corning) until 25–40% confluent. CM3 plasmid DNA (50 μg in 70 μl of TE, pH 7.8), and Calidria chrysotile (Gan et al., 1993) (500 μg in 1 ml of 140 mM NaCl, 25 mM HEPES, pH 7.4), were incubated together at 37°C for 30 min before adding to 9 ml of serum-free Ham's nutrient mixture F12 (J.R.H. Biosciences). The medium on the cells was aspirated and replaced by the chrysotile mixture. After 3 h, the mixture was aspirated, and the cells were washed twice with Ham's F12. Calcium phosphate transfections were done as described previously (Gan et al., 1993) with 50 μg of CM3 plasmid DNA, either untreated or γ-irradiated with a ^{60}Co source (total dose, 999.7 Gy), per 10 ml of precipitate suspension. Other controls were exposure to either 4.7 mM ethyl methanesulfonate (EMS, Sigma) or Calidria chrysotile (50 μg/ml; 6.7 μg/cm^2) without DNA. After the cells were treated, each flask was washed and cells propagated in Dulbecco's modified Eagle's medium (Bio Whittaker) containing 11% fetal bovine serum, penicillin and streptomycin (Gibco). Following exposure to test agents, cells were grown for 1.5 doublings and harvested. Cell pellets were stored at −70°C for mutational analysis.

For the mutagenesis assay, genomic DNA was prepared from treated or untreated Rat2λ cell pellets thawed and uniformly dispersed in 7 ml of 0.25 M sucrose with 20 mM Tris HCl and 10 mM EDTA with a Dounce homogenizer. The proteinase K digestion and subsequent phenol:chloroform extractions were performed as described (Shephard et al., 1993). Lambda shuttle vectors were rescued as infectious phage by exposing the genomic DNA, dissolved in Tris/EDTA, pH 7.4, to Transpack λ-packaging extracts as described by the supplier (Stratagene). 'Plating group' specifies genomic DNAs which were

packaged and plated on the same day. The phage were assayed for *lacI* mutations using previously described procedures (Mirsalis et al., 1993; Tinwell et al., 1994). Identified blue plaques were removed and the agar plugs were stored at 4°C in 500 μl of SM buffer with 20 μl of chloroform. Plaques were confirmed and purified by replating at low density. A blue plaque from a low density plate was selected and stored for sequence analysis (below). A packaging control experiment was done as described above except that a 5:1 ratio of untreated genomic Rat2λ DNA and CM3 plasmid DNA, respectively, was added to the λ-packaging extracts.

2.2. Sequence analysis

pLIZ plasmids were excised from isolated, repurified mutant (blue) phage plaques according to the manufacturer's instructions (ExAssist/SOLR System, Stratagene) using M13 helper phage and *E. coli* strains XL-1 Blue and SOLR. SOLR colonies containing pLIZ plasmids were selected by ampicillin resistance and grown overnight in 10 ml bacterial cultures from which plasmid mini preps were isolated by alkaline lysis (Sambrook et al., 1989). Plasmids were redissolved in 200 μl of TE, pH 7.4, containing DNase-free pancreatic RNase (Sigma), 20 μg/ml. Plasmids were then characterized by electrophoresis in agarose gels from which DNA concentrations were estimated. In sequencing reactions, plasmids were used as DNA template and an oligonucleotide complimentary to base pairs 848–865 of the *lacI* gene, 5′-GAC GAT ACC GAA GAC AGC-3′, was used as a primer, together with Taq polymerase and chain terminating, dye-labeled deoxyribonucleoside triphosphates. Reaction products were analyzed on an Applied Biosystems 373A automated sequencer (Smith et al., 1986; Connell et al., 1987; Halloran et al., 1993) which typically yielded 300–350 bases of readable sequence.

3. Results and discussion

The Rat2λ assay differs from conventional mammalian cell mutagenesis assays in three important respects: (1) Rat2λ cells (Wyborski et al., 1995)

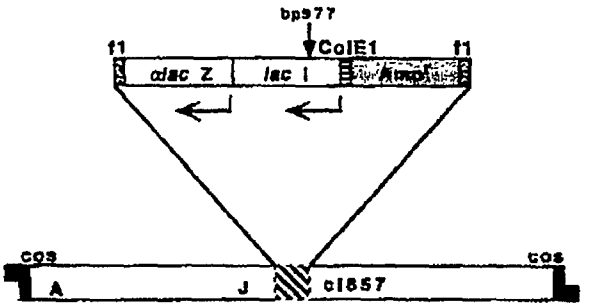

Fig. 1. The λ LIZ shuttle vector, modified from Wyborski et al. (1995), which contains the target for the mutagenesis assay. The λ LIZ construct (lower bar) contains a central cassette which can be excised as a phagemid, pLIZ (upper bar). The Rat2λ cell line (Big Blue rat cell line, Stratagene) carries 50–70 copies per cell of the modified λ LIZ vector stably integrated into the chromosomes at two distinct sites on separate chromosomes (Wyborski et al., 1995). The upper bar, flanked by f1 filamentous phage origins, represents that part of the vector which is excised (Mirsalis et al., 1993) as a phagemid, pLIZ, with ExAssist helper phage (Stratagene). The length of the DNA inserted into the λ arms was 4480 bp. Upon excision, however, the length of the excised pLIZ plasmid is 4334 bp (Wyborski, D.L., personal communication). The mutational target gene contained in pLIZ is the Lac repressor protein gene, *lacI*. Also contained in pLIZ is *αlacZ*, an ampicillin-resistance gene and a colE1 origin of replication. Control mutant 3 (CM3), provided by Stratagene as a reference standard, forms dark blue plaques on X-gal indicator plates and carries a point mutation of C → T at bp 977 (vertical arrow) in the *lacI* gene. This mutation converts a CAG codon (glutamine) to a TAG codon (stop).

contain multiple copies of the target gene, *lacI* (Fig. 1); (2) determinations of mutant frequencies are made in a prokaryotic format (a bacteriophage plaque assay) using DNA isolated from cells exposed to test mutagens in cell culture; and (3) mutants are identified not by metabolic selection, but by visual screening for mutant blue plaques amidst wild-type white plaques. The assay for homologous recombination is based on the following principle: Rat2λ cells are transfected with CM3 plasmid DNA (Fig. 1), which is a pLIZ plasmid containing a marker amber mutation in the *lacI* gene. The introduced mutant *lacI* gene can be subsequently recovered from the genomic DNA of the transfected cells by in vitro λ-packaging if, and only if, the introduced plasmid sequences have undergone recombination with homologous λ sequences residing in the Rat2λ genome (Kohler et al., 1991; Wyborski et al., 1995). In the absence of such recombination, the CM3 plasmid

cannot be recovered by in vitro packaging because it is too short and lacks λ *cos* sites. The region of homology between newly introduced and genomically integrated sequences extends over the entire length (4334 bp) of the CM3 plasmid, except for bp 977 of *lacI* (Fig. 1). If a fragment containing the marker mutation stop codon undergoes homologous recombination with one of the *lacI* genes in the Rat2λ genome, then that genomic *lacI* gene will subsequently produce dark blue plaques on agar containing X-gal. Once such a blue plaque is isolated, pLIZ can be excised and its *lacI* gene sequenced to determine if the CM3 marker mutation is present.

Rat2λ cells treated with chrysotile and CM3 plasmid DNA have a mutant frequency more than 3-fold higher than media or untreated controls (Table 1) and nearly half as high as cells treated with EMS, a potent mutagen which preferentially induces G:C → A:T transitions (Pienkowska et al., 1993). The media control and untreated cells show spontaneous mutant frequencies not significantly different from those reported previously (Wyborski et al., 1995). DNA from EMS-treated cells have a 6-fold increase in mutant frequency over the media control. Cells treated with chrysotile asbestos alone show a 2.5-fold increase over the media control. This value is significantly ($p < 0.05$) higher than the spontaneous mutant frequency. None of the samples exposed to calcium phosphate precipitation show a significant difference in mutant frequency indicating that transfection of the CM3 plasmid into the Rat2λ cells is not, in itself, sufficient to increase mutant frequency. Sequences of mutant *lacI* genes from recovered phage reveal the extent of asbestos recombinogenicity. More than half (53.8%) of the *lacI* sequences from mutant phage recovered from cells exposed to chrysotile + CM3 plasmid contain the CM3 marker mutation (C → T at bp 977, Fig. 1). Fig. 2 shows sequences of plasmids recovered from the mutagenesis assay. One plasmid has a wild-type sequence and the second plasmid contains the CM3 marker mutation. This point mutation is not seen in any sequence from other treatment groups. This indicates that asbestos promotes the recombination of the *lacI* gene bearing the marker mutation with the λ constructs residing in the Rat2λ genome. The results summarized in Table 1 demonstrate that a region of the

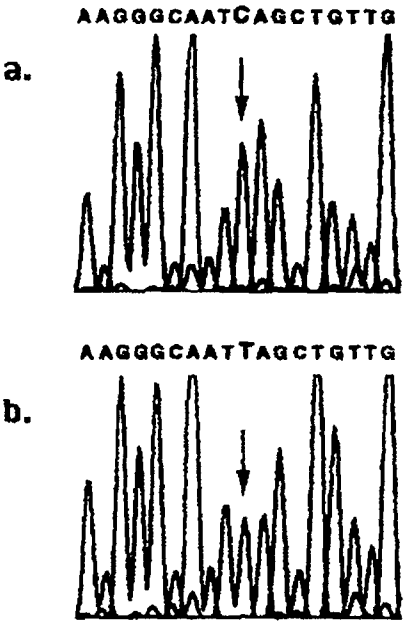

Fig. 2. Sequences of *lacI* (bp 968–985) from a wild-type plasmid (a) and a bp-977 C → T mutant plasmid (*b*). The plasmids were recovered from blue plaques identified in the mutagenesis assay. In this assay, identification of the marker mutation (C → T at bp 977) in a mutant plasmid is taken as evidence that a homologous recombination event occurred in the Rat2λ cell from which the mutant vector was recovered. Arrows indicate bp 977. The sequencing reaction products were analyzed on an automated sequencer (see Materials and methods).

CM3 plasmid containing the marker mutation has undergone homologous recombination in 7 of the 13 mutants derived from cells treated with chrysotile + CM3 plasmid.

It is important to raise the question of whether or not the seven mutants with the CM3 marker mutation reflect independent mutational events and to consider the possibility that they might have resulted from the expansion of a single mutant clone. In this experiment, following the 3-h exposure to test agent, the treated cultures were allowed to undergo 1.5 doublings; consequently, it is not possible to attribute all seven mutants to the postexposure expansion of a single mutant clone. The possibility that these seven mutants arose by a clonal expansion which occurred prior to exposure is effectively precluded by the failure to detect the marker mutation in any of the parallel control cultures all of which were seeded from the same starter culture shortly before exposure to test agents (Table 1).

Table 1
Induction of *lacI* mutations in Rat2λ cells and sequence analysis of selected mutant plaques

Treatment	Mutagenicity analysis [a]							Sequencing	
	Plating group	No. of blue mutants	No. of PFU	Mutant frequency [b]	Poissonian error [c]	*p*-value [d] (pg)	(t)	C → T bp 977 [e]	Total sequenced [f]
Media control	A	3	141000						
	B	12	212133						
	C	8	242890						
	D	2	190700						
	Total	25	675625	3.70	2.39; 5.29			0	9
EMS [g]	A	36	157050	22.92	16.06; 30.99	< 0.01	< 0.01	0	10
Chrysotile	A	11	121035	9.08	4.54; 15.19	< 0.01	< 0.05	0	4
Chrys. + CM3 [h]	B	23	178635	12.87	8.16; 18.65	< 0.01	< 0.01	7	13
Untreated	E	9	339045	2.65	1.21; 4.65	–	> 0.05	0	7
$CaPO_4$ + CM3 [i]	E	7	353280	1.98	0.80; 3.70	> 0.05	> 0.05	0	4
$CaPO_4$	F	10	368200	2.72	1.30; 4.64	–	> 0.05	0	8
$CaPO_4$ + CM3 (γ-irrad.) [j]	F	14	314250	4.46	2.44; 7.07	> 0.05	> 0.05	0	13
Pack. contr. [k]	G	28	524900	5.33	3.54; 7.48	–	> 0.05	0	14
									82

[a] Described in Materials and methods.
[b] Mutants/100000 plaque forming units (PFU).
[c] The deviation from the mean Poisson distribution.
[d] *p*-Value of the mean Poisson distribution of the sample mutant frequency compared to: pg, the mean Poisson distribution of the control sample done in the plating group; or t, the mean Poisson distribution of the media control 'total'. Statistical analysis was done as previously described (Shephard et al., 1993).
[e] Number of *lacI* sequences containing a C → T mutation at bp 977.
[f] Total number of recovered *lacI* genes sequenced.
[g] EMS, ethyl methanesulfonate.
[h] Chrys., Calidris chrysotile; CM3, the introduced plasmid DNA.
[i] $CaPO_4$, calcium phosphate precipitation.
[j] CM3 (γ-irrad.), γ-irradiated CM3 plasmid DNA.
[k] Packaging control experiment.

To test that the CM3 plasmid cannot be recovered by in vitro λ-packaging (i.e., that contaminating CM3 plasmids do not contribute to our results), the packaging reaction was performed on a mixture of CM3 plasmid DNA and genomic DNA from untreated cells. Of the fourteen mutant *lacI* genes isolated and sequenced, none have the marker mutation at bp 977 (Table 1). The result of this control is consistent with known requirements for in vitro λ-packaging and demonstrates that residual contaminating CM3 plasmids are not incorporated into λ-phage in the packaging reaction to any appreciable extent.

A total of 3240 base substitution mutations may be made in the 1080 base *lacI* gene. If we assume that 1800 of these base substitutions (55.5% of the total) can give rise to a detectable mutant and that all 1800 mutations occur at similar frequencies, then we may estimate the likelihood that the results obtained were due to chance. The probability that, among a set of thirteen spontaneously arising mutants, seven or more would carry the same specific base change at the same base is given by the expression:

$$p = \frac{\sum_{i=1}^{7} (1800)_i}{1800^{13}}$$

$$p = 2.91 \times 10^{-20}$$

where $(1800)_i = 1800 \cdot (1799) \cdots (1800 - i + 1)$. This equation describes the probability that *any* mutation would occur seven or more times among a set of 13 independent mutants. It does not take into account the fact that the mutation recovered multiple times was indeed identical to the marker mutation present on the transfected *lacI* gene. Nor does it account for the fact that the marker mutation at base 977 is situated in a 31-base zone (960–990) in which detectable mutations occur only sparsely. Lastly, this calculation considers only base substitution mutations and neglects the contributions of small and large deletions and insertions to the totality of possible *lacI* mutations.

In light of this exceedingly low probability, we conclude that the seven marker mutations occurred, not by chance, but instead by a facilitated process, namely homologous recombination.

Classical tumor promoters are operationally ineffective at tumor induction when applied alone or before an initiator (mutagen). This lack of productivity and apparent lack of permanence has fostered the conventional view that all tumor promoters must enhance carcinogenesis by epigenetic, non-genotoxic mechanisms. This view, however, has been difficult to reconcile with the strong association of tumor promotion with oxygen radical formation (Cerutti, 1985) and DNA strand breaks (Birnboim, 1983) which are potentially and explicitly genotoxic, respectively. Any agent which can increase the frequency of mitotic recombination events can cause the expression of recessive mutations at tumor suppressor loci, and by this mechanism act as a tumor promoter (Kinsella and Radman, 1978). The ability of mitotic recombination events to act as tumor promoters is absolutely contingent on prior exposure to mutagen (initiator) which is required to create recessive mutations at tumor suppressor loci (Passarge and Bartram, 1976; Kinsella and Radman, 1978; Cavenee et al., 1983; Knudson, 1986). This dependence on prior initiation is completely concordant with the classic concepts of tumor promotion (Berenblum, 1975).

Both et al. (1995) have recently reported that treatment of human mesothelioma cells in culture with crocidolite asbestos fibers induces an increased frequency of loss of heterozygosity (LOH) at two loci on chromosome 6. These authors attribute this asbestos-induced LOH to either of two mechanisms: mitotic recombination or very large deletions. Homologous recombination events are likely to play a pivotal role in both of these mechanisms.The frequently observed synergism of asbestos fibers with other carcinogens has prompted classification of these fibers as tumor promoters. Moreover, asbestos fibers display two additional stigmata of tumor promoters, namely, enhancement of oxygen radical formation (Kamp et al., 1992) and induction of DNA strand breaks (Libbus et al., 1989; Kamp et al., 1995). Our present results indicate an ability of asbestos fibers to increase the frequency of recombination events between homologous sequences within the nucleus and suggest a genetic mechanism for the promoter-like synergism of asbestos with other mutagens (Hei, 1991) and carcinogens (Selikoff et al., 1968; Jaurand, 1989; Fasy, 1991; Walker et al., 1992). Most

asbestos-induced mitotic recombination events would be inconsequential in the absence of prior mutations on the recombining chromosomes. Following exposures to other mutagens, however, the recombinogenicity of asbestos may acquire enhanced mutagenic, and carcinogenic, potential.

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