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The Mutant Frequencies and Types of Mutations Induced by Comfrey in the Lungs of Transgenic Big Blue Rats

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(The views presented in this article do not necessarily reflect those of the U.S. Food and Drug Administration.)

ABSTRACT

Comfrey (*Symphytum Officinale*) has been used as a vegetable and herbal remedy for many years. It, however, is a mutagen and carcinogen in liver and a possible genotoxin in lung. In order to evaluate the mutagenicity of comfrey and the mechanisms of action in rat lung, we examined the mutant frequencies (MF) and mutational types in the lung *cII* gene from transgenic Big Blue rats treated with 8% comfrey for 12 weeks. The *cII* MF in the lungs for rats fed with comfrey was $48 \pm 9 \times 10^{-6}$, which was significantly greater than the MF for control rats, $34 \pm 10 \times 10^{-6}$ ($p = 0.026$). The mutational spectrum in lung from comfrey-fed rats is significantly different from that in the control. G:C → T:A transversion (29%) was the major type of mutation in comfrey-fed rats, whereas G:C → A:T transition (63%) was the predominant mutation in the controls. An unusual type of tandem–base substitution (4%) was observed among the mutations from comfrey-fed rats that were not seen in the control. This type of tandem-base substitution has been suggested as a mutational signature for the genetic damage of pyrrolizidine alkaloids (PAs). The results indicate that comfrey is mutagenic in rat lung and the types of mutations induced by comfrey are similar to those by other PAs; and suggest that the mutagenicity of comfrey in rat lung results from the genotoxicity of PAs in the plant.

Key words: comfrey, pyrrolizidine alkaloids, mutation, tandem substitution

INTRODUCTION

Comfrey (*Symphytum Officinale*) grows in rich, moist, low meadows or along river banks, where it can reach a height of four feet[(1)]. Comfrey is a popular dietary supplement and herbal remedy used for healing broken bones and for treating ulcers, bruises and digestive tract upsets in many cultures, and is cultivated for use as a green vegetable or tonic in Japan[(2,3)]. It, however, has been reported that comfrey is hepatotoxic in livestock and humans, and carcinogenic in experimental animals. In Russia, the herb is considered poisonous when used excessively. It has also been linked to cases of veno-occlusive disease in the last 20 years[(4)]. After taking comfrey, patients presented with hepatic veno-occlusive disease[(5)] and severe portal hypertension and subsequently died from liver failure[(6)]. Rats that received a diet containing comfrey roots and leaves developed hepatocellular adenomas[(2)]. Acetone extracts from comfrey produced mutations in *Salmonella* without microsomal bioactivation using strains TA98 and TA100[(7)]. Recently, we demonstrated that comfrey was a mutagen in rat liver[(8)].

Comfrey roots and leaves contain about 9 pyrrolizidine alkaloids (PAs)[(9)]. The major unsaturated PAs in comfrey are the monoesters lycopsamine and intermedine, their acetyl derivatives (7-acetyllycopsamine and 7-acetylintermedine), and symphytine[(10,11)]. Unsaturated PAs are metabolically activated to toxic compounds in the liver by mixed function oxidases. This process involves dehydrogenation of the pyrrolizidine to form the corresponding pyrrole, a potent alkylating agent[(11)]. PAs can be toxic to many different organs. The most frequently observed toxicity is to the liver. In addition, PAs have also been noted to cause damage in the lungs and kidneys[(12)]. The presence of PAs in comfrey and the widespread use of comfrey in foods and drinks raise questionable concerns[(13)]. The use of comfrey as a dietary supplement and in herbal medicinal products intended for ingestion has been banned in Germany, Canada, and the United States[(9,14,15)]. Although comfrey has been banned in several countries, it is still are available in many other countries or through internet ordering. Thus, the safety of comfrey, a widely used herb in relation to human consumption, requires further investigation, especially its mutagenicity and carcinogenicity.

Rodents have long been used as animal models to predict and estimate the risk or effects associated with mutagens and carcinogens in the human population, and the testing of rats with comfrey serves a similar purpose in this study to predict the mutagenic effects of comfrey

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in rat lung. Big Blue transgenic mutation models provide a tool for studying mutant frequencies and types of mutations in nearly all tissues, thus permitting the identification of mutagenicity in specific tissues[16-18]. In these systems, the chromosomally-integrated *cII* gene located on shuttle vectors that are derivatives of the bacteriophage lambda (λ) is used as the target for mutation. Multiple genomic copies of the phage are contained within the genome of each transgenic animal cell as stably integrated concatamers. After exposure of transgenic animals to a test substance, DNA can be isolated from individual organs, and single copies of the phage genome can be excised from the high molecular weight DNA and packaged into infectious virus particles with the help of a packaging mix[19]. If appropriate *E. coli* host cells are infected by the packaged virus, plated in culture medium, and incubated in an appropriate temperature, plaques will become visible on the plates within hours. *cII* Mutant plaques can be selected and identified through a lower culture temperature (24°C) than that for normal plaque formation (37°C).

The objective of this study is to evaluate the mutagenicity of comfrey in rat lung and to determine whether or not PAs in comfrey are the main components responsible for the genotoxicity. To do so, Big Blue transgenic rats were treated with comfrey and the mutant frequency (MF) induced by comfrey was determined in the *cII* gene of lungs. The mutational spectra from control and comfrey-induced mutants were also determined to examine the mutational signature of PAs.

MATERIALS AND METHODS

I. *Animals and Treatments*

Big Blue transgenic rats were purchased from Taconic Laboratories (Germantown, NY). The treatment schedule of comfrey was based on a previous study that evaluated the carcinogenicity of comfrey[2]. Comfrey roots were obtained from Camas Prairie Products (Trout Lake, WA). PAs in the comfrey roots were determined by mass spectral analysis of an extract. The PAs detected were similar to those reported previously[13], and included symphytine, 7-acetyllycopsamine, and 7-acetylintermedine as major components in near equal amounts; intermedine and lycopsamine were present in a relatively smaller quantity. To determine a dose for treatment of comfrey, a preliminary experiment was conducted with different doses. A diet containing 8% comfrey root was chosen for this experiment according to the toxic effect on weight gain and lack of overt toxicity. The comfrey roots were ground and then blended with basal diet powder in a Hobart Mixer to make an 8% comfrey root diet. Groups of six 6-week-old male Big Blue rats were fed either a normal diet or the comfrey diet. The animals were sacrificed one day after 12-week treatment. The recommendations set forth by our Institutional Animal Care and Use Committee for the handling, maintenance, treatment, and sacrifice of the animals were followed. The lungs were isolated, frozen quickly by using liquid nitrogen, and stored at -80°C.

II. *Isolation of DNA from Lung Tissue*

RecoverEase DNA isolation kit was purchased from Stratagene (La Jolla, CA) for DNA isolation. Each DNA sample was isolated from ~100mg of lung tissue. The samples were gently homogenized in 5 mL cold lysis buffer to disaggregate, and then sent through a sterile cell strainer into a 50-mL conical tube to be centrifuged at 1100 ×g for 12 min at 4°C. The supernatant was discarded and a fresh 10 mL of cold lysis buffer was added for another centrifugation at 1100 ×g for 5 min at 4°C. The supernatant was again discarded and the residual droplets were removed with a sterile applicator. A digestion solution was composed with a 20 μL RNace-It ribonuclease cocktail in 1 mL of digestion buffer. Seventy microliter of this digestion solution and 70 μL of warmed proteinase K solution was mixed and added into the pellet. The conical tube was then placed in a 50°C water bath for 45 min. The genomic DNA was then transferred to a dialysis cup floating on TE buffer prepared in advance, and left to dialyze at room temperature for approximately 25 hr. The fully hydrated genomic DNA was then removed from the dialysis cup and stored at 4°C until use.

III. *cII Mutation Assay*

The packaging of the phage, plating of the packaged DNA samples, and determination of MF were carried out following Stratagene's procedure for the λ select-*cII* mutation detection system for Big Blue rodents. The shuttle vector containing the *cII* target gene was rescued from total genomic DNA with phage packaging extract (Transpack; Stratagene). The plating was performed with the *Escherichia coli* host strain G1250. The bacteria were grown in TB1 liquid medium with 1% maltose-$MgSO_4$ (1 M) overnight at 30°C in preparation for the experiment. To determine the total titer of packaged phages, G1250 bacteria were mixed with a 1:3000 dilution of phage, plated on TB1 plates, and incubated overnight at 37°C (nonselective conditions). For mutant selection, the packaged phages were mixed with G1250, plated on TB1 plates and incubated at 24°C for about 42 hr (conditions for *cII*-selection). The *cII* MF was calculated as the ratio of the total number of mutant plaques (as determined at 24°C) to the total number of plaques screened (as determined at 37°C).

IV. *Sequence Analysis of the cII Mutants*

The sequencing protocol was adapted from previous procedure[20,21]. The *cII* mutant plaques were selected at random from different animals and replated at low densi-

ty to verify the mutant phenotype. Single, well-isolated plaques were selected from these plates and transferred to a microcentrifuge tube containing 50 μL of autoclaved distilled water. The tubes were placed in a thermocycler at 99.9°C for 5 min and centrifuged at 1500 ×g for 5 min immediately after the heating. The *cII* target DNA was amplified by PCR with primers reported previously[21]. For PCR amplification, 10 μL of the supernatant and 0.03 μL of the each primer (148 μM) were added to 10 μL of 2× PCR Master Mix (Promega, Madison, WI). The PCR reaction was run with the following cycling parameters: a 3 min denaturation at 95°C; followed by 30 cycles of 30 sec at 95°C, 1 min at 60°C, and 1 min at 72°C; with a final extension of 10 min at 72°C. The PCR products were purified using PCR purification kits (Qiagen, Chatsworth, CA). The *cII* mutant DNA was sequenced and analyzed with a CEQ DTCS-Quick Start Kit and a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions. The primers for *cII* mutation sequencing were the same as those used for the PCR.

V. *Statistical Analyses*

Statistical analyses of MFs were performed using SigmaStat (SPSS Science, Chicago, IL). All MF data are expressed as the mean ± standard deviation (SD) from 6 different animals. Statistical significance was determined by student t-test for comparison of the control and treatment groups. The difference of mutational types between different groups was paired and tested statistically using the computer program written by Cariello et al.[22] for the Monte Carlo analysis developed by Adams and Skopek[23].

RESULTS

The sample groups containing 6 male Big Blue rats were administered 8% comfrey root for 12 weeks and sacrificed one day after administration with corresponding controls. The MFs in the lung *cII* gene were determined (Table 1). The MF for rats fed with comfrey was $47.7 \pm 8.9 \times 10^{-6}$, which showed a significant increase in MF when compared to their control, $33.8 \pm 9.6 \times 10^{-6}$ ($p = 0.026$). Mutational types from the control and comfrey-induced mutants were listed in Table 2 and summarized in Table 3. Statistical evaluation of the two spectra indicates that the spectrum from comfrey-fed rats is significantly different from the control. G:C → T:A transversion (29%) was the major type of mutation in comfrey-fed rats, whereas G:C → A:T transition was the predominant mutation in the controls. An unusually high frequency of A:T → T:A transversion (16%) and tandem–base substitution (4%) were observed among the mutations from comfrey-fed rats while no such mutations were found in the control rats.

DISCUSSION

The mutagenicity test of comfrey in rat lung showed significantly positive, indicating that comfrey is a rat lung mutagen. Compared to liver[8], the MF in lung was significantly less. A dose of 2% comfrey induced about 140×10^{-6} MF in liver, about 3-fold higher than that in lung. Although it is very clear that PAs can be metabolized to active mutagens in liver, we do not know whether or not lung can metabolize PAs efficiently like liver. Although it is possible for metabolites of PA to reach to

Table 1. Lung *cII* mutant frequencies in the comfrey-treated and control Big Blue rats

Group	Rat ID	Total plaques screened ($\times 10^3$)	Mutant plaques	Mutant frequency ($\times 10^{-6}$)
Control	1 M	418	21	50.2
	2 M	993	35	35.2
	3 M	484	18	37.2
	4 M	888	27	30.4
	5 M	461	12	26.0
	6 M	1108	26	23.5
	Mean ± SD			33.8 ± 9.6
Comfrey	1 M	566	26	45.9
	2 M	766	44	57.5
	3 M	335	18	53.7
	4 M	979	37	37.8
	5 M	649	24	37.0
	6 M	587	32	54.5
	Mean ± SD			47.7 ± 8.9*

*Significantly higher than the control group ($p = 0.026$).

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Table 2. Types of mutations in the lung *cII* gene from comfrey-treated and control Big Blue rats

Position[a]	Mutation[b]	Amino acid change	Sequence context 5' → 3'[c]	Number of mutations (independent)	
				Control	8% Comfrey
-14	G → T	N/A	ctaAGGaaa		1
	G → A	N/A	ctaAGGaaa	1	
-13	G → T	N/A	ctaAGGaaa		2 (2)
3	G → T	Met → Ile	catATGgtt	1	1
	G → A	Met → Ile	catATGgtt	2 (2)	
4	G → C	Val → Leu	atgGTTcgt		1
19	C → T	Arg → Cys	aaaCGCaac	2 (2)	1
25	G → A	Glu → Lys	aacGAGgct	1	
	G → T	Glu → Stop	aacGAGgct		2 (2)
26	A → G	Glu → Gly	aacGAGgct		1
29	C → T	Ala → Val	gagGCTcta	1	
34	C → T	Arg → Stop	ctaCGAatc	3 (2)	
35	G → A	Arg → Gln	ctaCGAatc		1
40	G → A	Glu → Lys	atcGAGagt	1	1
41	A → G	Glu → Gly	atcGAGagt		1
50	T → A	Leu → Stop	gcgTTGctt		1
	T → C	Leu → Ser	gcgTTGctt	1	1
52	C → T	Leu → Phe	ttgCTTaac		2 (2)
57	C → G	Asn → Lys	cttAACaaa		1
64	G → A	Ala → Thr	atcGCAatg	1	3 (1)
	G → C	Ala → Pro	atcGCAatg	1	
73-74	GG → TT	Gly → Leu	cttGGAact		1
89	C → T	Ala → Val	acaGCGgaa	2 (2)	2 (2)
94	G → T	Ala → Ser	gaaGCTgtg	1	
95	C → T	Ala → Val	gaaGCTgtg	1	
100	G → A	Gly → Ser	gtgGGCgtt	1	
	G → T	Gly → Cys	gtgGGCgtt		1
101	G → A	Gly → Asp	gtgGGCgtt		1
	G → T	Gly → Val	gtgGGCgtt		1
102	C → T	Gly → Gly	gtgGGCgtt	1	
103	G → A	Val → Ile	ggcGTTgat	6 (5)	3 (3)
	G → T	Val → Phe	ggcGTTgat		2 (2)
103-104	GT → TA	Gly → Tyr	ggcGTTgat		1
113	C → T	Ser → Leu	aagTCGcag	2 (2)	1
117	G → T	Gln → His	tcgCAGatc	1	1
	G → C	Gln → His	tcgCAGatc		1
119	T → A	Ile → Asn	cagATCagc		1
	T → C	Ile → Thr	cagATCagc		1
122	G → A	Ser → Asn	atcAGCagg		1
125	G → C	Arg → Thr	agcAGGtgg		1
128-129	- G	Frameshift	aggTGGaag	1	
129	G → T	Trp → Cys	aggTGGaag	1	1
132	G → T	Lys → Asn	tggAAGagg		1
134	G → T	Arg → Met	aagAGGgac		1
	G → C	Arg → Thr	aagAGGgac		1

Table 2. (Continued)

Position[a]	Mutation[b]	Amino acid change	Sequence context 5' → 3'[c]	Number of mutations (independent)	
				Control	8% Comfrey
135	G → T	Arg → Ser	aagAGGgac		2 (2)
134-136	- G	Frameshift	aagAGGGACtgg	1	
140	G → T	Trp → Leu	gacTGGatt		1
	G → C	Trp → Ser	gacTGGatt	1	
142	A → T	Ile → Phe	tggATTcca		1
143	T → A	Ile → Asp	tggATTcca		1
147-149	- A	Frameshift	attCCAaag	1	
152	T → A	Phe → Tyr	aagTTCtca		1
154	T → A	Ser □Thr	ttcTCAatg		1
155	C → A	Ser → Stop	ttcTCAatg	1	
161	T → A	Leu → Gln	atgCTGctt		2 (2)
163	- C	Franeshift	ctgCTTgct	1	
164	T → G	Leu → Arg	ctgCTTgct		1
169	G → C	Val → Leu	gctGTTctt	1[d]	
170	T → G	Val → Gly	gctGTTctt		1
172	C → T	Leu → Phe	gttCTTgaa	1	
173	T → A	Leu → His	gttCTTgaa		1
	T → C	Leu → Pro	gttCTTgaa		1
175	G → C	Glu → Glu	cttGAAtgg		1
178-185	+ G	Frameshift	gaaTGGGGGGTCgtt	1	4 (2)
179-184	- G	Frameshift	gaaTGGGGGGTCgtt	1	1
180	G → A	Trp → Stop	gaaTGGggg		1
180-181	GG → TT	TrpGly → CysTrp	gaaTGGGGGgtc		1
187	G → T	Val → Phe	gtcGTTgac		2 (2)
191	A → C	Asp → Ala	gttGACgac		1
193	G → A	Asp → Asn	gacGACgac		1
196	G → A	Asp → Asn	gacGACatg	1[d]	3 (2)
	G → C	Asp → His	gacGACatg	1	
197	A → T	Asp → Val	gacGACatg		1
200	T → A	Met → Lys	gacATGgct		1
202	G → T	Ala → Ser	atgGCTcga		1
206	G → A	Arg → Gln	gctCGAttg	1	1
210	G → T	Leu → Phe	cgaTTGgcg		1
212	C → A	Ala → Glu	ttgGCGcga		1
	C → T	Ala → Val	ttgGCGcga	4 (3)	1
214	C → T	Arg → Stop	gcgCGAcaa	6 (3)	1
217	C → T	Gln → Stop	cgaCAAgtt		1
218	A → T	Gln → Leu	cgaCAAgtt		1
220	G → T	Val → Phe	caaGTTgct		1
221	T → G	Val → Gly	caaGTTgct	1	
226	G → C	Ala → Pro	gctGCGatt	1	
232	C → G	Leu → Val	attCTCacc	2 (1)	
233	T → A	Leu → Tyr	attCTCacc		1
240	- T	Frameshift	accAATaaa		1
241-246	- A	Frameshift	aatAAAAAAcgc	1	

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Table 2. (Continued)

Position[a]	Mutation[b]	Amino acid change	Sequence context 5' → 3'[c]	Number of mutations (independent)	
				Control	8% Comfrey
249-251	- C	Frameshift	aaaCGCCCGgcg	1	
274	C → G	Gln → Glu	gaaCAAatc		1
293	G → T	Stop → Leu	ttcTGAggt		1
Total				59 (52)	86 (81)

[a]Position 1 is the first base of the start codon in the *cII* coding sequence.
[b]Presented in term of sequence change on nontranscribed DNA strand.
[c]Uppercase indicates target codon and target bases are underlined.
[d]Indicates the two mutations generated from a same mutant.
Abbreviations: -, deletion; +, insertion.

Table 3. Summary of the independent mutations in the lung *cII* gene from comfrey treated and control Big Blue rats

Type of mutation	Control		Comfrey*	
	Number	%	Number	%
G:C → C:G	5	9	7	8
G:C → A:T	33	63	23	28
G:C → T:A	5	9	24	29
A:T → T:A	0	0	13	16
A:T → C:G	1	2	3	4
A:T → G:C	1	2	5	6
Frameshifts	7	13	4	5
Complex mutation	1	2	0	0
Tandem–base substitution	0	0	3	4
Total mutants screened	53	100	82	100

* Spectra for comfrey treated rats were significantly different from the controls ($p < 0.0001$).

lung via blood circulation, it is nearly impossible for the active metabolite, 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) that results in PA-DNA adducts and mutations to move from one organ to another without binding to molecules like proteins, DNA and RNA due to its strong activity and instability[24,25]. Metabolism of retrorsine, a PA, with lung microsomes from dexamethasone-treated rats generated DHP[26], suggesting that lung may possess the capability of metabolizing PAs. Several PAs can induce hypertensive pulmonary vascular disease[27-29]. Monocrotaline, a pyrrolizidine alkaloid, causes veno-occlusive disease of the liver, pulmonary arterial hypertension, and right ventricular hypertrophy. Its toxicity is proven due to formation of a pyrolic metabolite that can be detoxified by conjugation with glutathione (GSH). This PA has been used to compare pyrrolic metabolites of PA in liver and in lung by measuring GSH level. Higher levels of the metabolites were found in liver than in lung[30,31], indicating that the lung is a secondary target of PAs subsequent to the liver. Our mutagenicity data are correlated with these metabolic studies.

The liver histology of comfrey-treated rats is quite similar to that produced by some hepatotoxic PAs[32-34] and our previous studies also suggested that PAs in comfrey are responsible for the mutagenicity and carcinogenicity in rat liver[8,35]. Comfrey contains up to nine PAs[14,36,37], at least two of which, symphytine and lasiocarpine, have been proven to be carcinogenic[38,39]. It is still unclear whether or not the PAs in comfrey respond for the mutagenicity of comfrey in rat lung. To understand the mechanism for induction of mutations by comfrey in lung, we sequenced the DNA from the mutants generated from the comfrey-treated and control rats to determine whether the types of mutations induced by comfrey showed the signature of PAs. We investigated the mutagenicity of riddelliine, a representative genotoxic PA, in Big Blue rat liver and found that riddelliine induced a major type of mutation, G:C → T:A transversion and an unusually high frequency of tandem base substitution[40]. The types of mutations induced by comfrey in rat liver were also determined[8]. G:C → T:A transversion was the major type of mutation in comfrey-fed rats and an unusually high frequency of tandem base substitutions (17%) was observed among the mutations from comfrey-fed rats. Since it has been suggested that all PAs produce the same type of DNA adducts[41], we would expected that all PAs would produce similar types of mutations. Therefore, G:C → T:A transversion and tandem base substitution has been suggested as a mutational signature for the genetic damage of PAs[8,40]. In this study, G:C → T:A transversion as the main type of mutations and the tandem-base substitutions were observed among the comfrey-induced mutations. These signature mutations indicate that mutations induced by comfrey in rat lungs are due to PAs in the plant. Besides, an unusually high frequencies of A:T → T:A transversion that was not found in the comfrey-induced mutations in rat liver and in the spontaneous mutations, which is not able to be explained by PAs' toxicity, suggests that mechanism(s) other than PAs might involved in the

mutagenicity of comfrey in rat lung. Since PA has been linked to liver and lung cancers and a range of other deleterious effects[42,43], lung is a possible carcinogenic target for comfrey.

In summary, treatment of transgenic Big Blue rats with comfrey induced mutations in the lung *cII* gene. The mutational spectrum from comfrey-treated rats suggests that PAs in the plant are mainly responsible for the mutation induction in rat lungs. Therefore, comfrey is a rat lung mutagen and may present a health risk to human population for pulmonary diseases.

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