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The Genotoxicity of Mainstream and Sidestream Marijuana and Tobacco Smoke Condensates

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While the prevalence of tobacco use has decreased in Canada over the past decade, that of marijuana use has increased, particularly among youth. However, the risks of adverse health effects from marijuana smoke exposure, specifically as compared to tobacco, are currently not well understood. The objectives of this study were to evaluate the relative ability of matched marijuana and tobacco condensates to induce (geno)toxic responses in three in vitro test systems. This study provides comparative data for matched sidestream and mainstream condensates, as well as condensates prepared under both a standard and an extreme smoking regime designed to mimic marijuana smoking habits. The results indicate that tobacco and marijuana smoke differ substantially in terms of their cytotoxicity, *Salmonella* mutagenicity, and ability to induce chromosomal damage (i.e., micronucleus formation). Specifically, the marijuana condensates were all found to be more cytotoxic and more mutagenic in the presence of S9 than the matched tobacco condensates. In contrast, the tobacco condensates appeared to induce cytogenetic damage in a concentration-dependent manner, whereas the matched marijuana condensates did not. In addition, when corrected for total particulate matter yield, little difference was observed in the mutagenic activity of samples smoked under the extreme vs the standard regime for both tobacco and marijuana condensates.

Introduction

The prevalence of marijuana use is high among Canadian youth aged 15−24 years with about 25% of them in 2006 having reported using marijuana at least once in the previous 12 months; among those, 14.3% used marijuana daily, which represents 3.1% of all Canadian youth (*1*). In comparison, 14% of Canadian youth reported smoking tobacco cigarettes daily and 7.6% occasionally.

Marijuana is often viewed as a "natural" product, and marijuana smoking is often perceived as less harmful than smoking tobacco. To date, neither marijuana plant extracts nor Δ^9-tetrahydrocannabinol (THC), the main psychoactive component of marijuana, have been confirmed to be mutagenic or carcinogenic on their own (*2*−*5*). However, it is generally acknowledged that, like tobacco, marijuana smoke contains harmful substances that likely stem from the pyrolysis of the plant material (i.e., the smoking process). Our earlier companion study constituted a systematic comparison between the chemical composition of mainstream/sidestream marijuana smoke and mainstream/sidestream tobacco smoke generated from cigarettes prepared under the same conditions. That work confirmed that marijuana smoke condensates contain qualitatively the same chemicals as tobacco smoke (*6*). Of the thousands of chemicals that have been identified in tobacco smoke condensate by ours and other studies, at least 81 of these have been classified as carcinogens by the International Agency for Research on Cancer (IARC) (*7*). Table 1, including an excerpt of the chemical data presented in our earlier study, shows the concentrations of a select number of carcinogenic and/or mutagenic chemicals in detected and quantified mainstream tobacco and marijuana smoke.

Like tobacco smoke, marijuana smoke has been associated with numerous adverse pulmonary effects in humans including chronic bronchitis, edema, and mucus hypersecretion (*8*). Indeed, a recent study comparing the pulmonary effects of marijuana and tobacco smokes in humans noted that the impairment of large airway function and lung efficiency induced by marijuana smoke was 2.5−5 times greater than that observed for tobacco smoke (*9*). In addition, several previous studies have shown that marijuana smoke condensates are genotoxic. Early in vitro studies demonstrated that marijuana smoke condensates are mutagenic to bacteria in the *Salmonella* reverse mutation assay (*2, 10*), and whole marijuana smoke can alter DNA content and chromosome number in human lung explants (*11*). In human studies, researchers have noted an increase in *HPRT* gene mutations in the lymphocytes of marijuana-smoking mothers and their newborns (*12*) and an increase in DNA strand breaks in macrophages lavaged from the lungs of marijuana smokers (*13*). Although confounded by concurrent tobacco use, other in vivo studies have found higher levels of chromosomal breaks (*14*), chromosomal damage (*15*), and sister chromatid exchanges (*16*) in marijuana users as compared to controls.

Despite these findings, the limited number of epidemiological studies (i.e., small case control studies) conducted to date have yielded conflicting results (often confounded by concurrent tobacco use) and have thus far failed to establish a convincing link between marijuana smoking and the development of respiratory cancers (*17*−*21*). However, it should be noted that insufficient marijuana-only smoking populations have been

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Table 1. Levels of Selected Carcinogens and/or Mutagens in Mainstream Tobacco and Marijuana Smoke Condensates Generated under Two Smoking Conditions[a]

	ISO[c]		extreme[d]		IARC	
analyte[b]	tobacco	marijuana	tobacco	marijuana	carcinogenicity[e]	mutagenicity[f]
benzo[*a*]anthracene	0.611	0.504	0.458	0.332	2A	1, 2, 3
benzo[*b*]fluoranthene	0.217	0.138	0.193	0.125	2B	1
benzo[*k*]fluoranthene	0.069	0.029	0.066	0.035	2B	1
benzo[*a*]pyrene	0.287	0.166	0.221	0.119	1	1, 2, 3
indeno[1,2,3-*cd*]pyrene	0.092	0.069	0.089	0.067	2B	1
dibenz[*a*,*h*]anthracene	0.023	0.027	0.043	0.022	2A	1, 2
benzo[*g*,*h*,*i*]perylene	0.076	0.049	0.063	0.046	3	1
benzo[*j*]fluoranthene	0.116	0.082	0.117	0.094	2B	1
dibenz[*a*,*i*]pyrene	0.020	NQ	0.022	BDL	2B	1
4-aminobiphenyl	0.031	0.118	0.022	0.104	1	1, 2
NNN	1.756	BDL	1.408	BDL	1	1
NNK	1.737	BDL	1.391	BDL	1	1, 2
cadmium	2.906	0.133	2.500	0.112	1	1, 2
lead	0.423	NQ	0.386	NQ	2B	0
arsenic	0.110	NQ	0.112	NQ	1	1
formaldehyde†	4.008	0.482	4.780	0.512	2A	1, 2, 3
acetaldehyde†	17.475	8.599	13.688	7.854	2B	1, 2, 3
1,3-butadiene†	1.299	1.526	1.092	1.062	2A	1, 2, 3
isoprene†	5.731	1.420	4.754	1.015	2B	0
acrylonitrile†	0.261	0.702	0.211	0.515	2B	1, 2, 3
benzene†	1.246	1.119	0.833	0.649	1	1, 2, 3
styrene†	0.301	0.330	0.252	0.344	2B	1

[a] NNN, *N*′-nitrosornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; BDL, below detection level; NQ, not quantifiable (above the limit of detection but below the limit of quantitation). [b] Units are in ng/mg total particulate matter (TPM). Analytes flagged with † are in μg/mg TPM. [c] ISO, International Organization for Standardization standard 3308. [d] Modified smoking regime designed to reflect marijuana smoking habits. See the text for details. [e] On the basis of IARC monographs 29, 32, 58, 71, 82, 84, 87, 88, 89, 92 (in preparation), and supplement 7. Key: 1, carcinogenic to humans; 2A, probably carcinogenic to humans; 2B, possibly carcinogenic to humans; 3, inadequate or limited evidence of carcinogenicity in experimental animals (available online at http://monographs.iarc.fr). [f] On the basis of IARC monographs 29, 32, 58, 71, 82, 84, 87, 88, 89, 92 (in preparation), and supplement 7. Key: 0, no evidence for mutagenicity; 1, mutagenic in bacterial and/or fungal/yeast cells in vitro; 2, mutagenic in plants or animals in vitro; 3, mutagenic in *Drosophilia melanogaster* somatic mutation and recombination test and/or sex-linked recessive lethal test and/or transgenic rodent assay and/or rodent dominant lethal test (available online at http://monographs.iarc.fr).

studied to date, and this may account for the difficulty of establishing a statistically defensible association between marijuana smoking and respiratory cancers. It took decades of study to establish the empirical and mechanistic links between tobacco smoke exposure and lung cancer, despite the fact that tobacco-only smokers, nonsmokers, and former smokers could readily be identified (*22*).

It has been suggested that differences in the pharmacological properties of marijuana smoke, as compared to tobacco smoke, may be responsible for the lack of correlation between marijuana smoking and respiratory cancers. For instance, it has been observed that unlike marijuana smoke, tobacco smoke prevents apoptosis, thereby allowing genetically damaged cells to survive and replicate. Moreover, nicotine promotes tumor angiogenesis while cannabinoids support tumor regression, and the THC in marijuana smoke inhibits cytochrome CYP1A1 function, thus guarding against metabolic activation of selected carcinogens [e.g., polycyclic aromatic hydrocarbons (PAHs), nitrosamines] (*23*).

Clearly, the risks of genotoxic effects from marijuana smoke exposure, specifically as compared to tobacco, are currently not well understood, and this type of information is crucial for effective public health policies and, moreover, the communication of risk to marijuana users and youth in particular. This study is a continuation of the aforementioned earlier work that examined the chemical composition of tobacco and marijuana smoke condensates. This companion study presents the results of the toxicological assessments. The objectives of this study were to evaluate the genotoxicity of mainstream and sidestream marijuana smoke condensates, as compared to mainstream and sidestream tobacco smoke condensates. To ensure accurate comparisons of the two products, the tobacco and marijuana cigarettes were prepared and combusted under identical conditions. In addition, a modified smoking condition, designed to reflect marijuana smoking habits, was evaluated in comparison to a standard tobacco smoking regime.

Materials and Methods

Smoking Procedure. The tobacco samples consisted of Player's fine-cut loose tobacco obtained from a local retail store. The marijuana samples consisted of a standardized product, containing only flowering heads. The product was obtained from Prairie Plant Systems Inc. (Saskatoon, Canada), the government of Canada's contracted grower of medicinal marijuana, and all samples were from harvest #55 (May 2004, reference H55-MS17/338-FH). Upon harvest, flowering heads were dried to a moisture content of approximately 10%, milled to 10 mm, packaged, and irradiated.

The preparation and combustion of the marijuana and tobacco cigarettes were conducted by Labstat International Inc. (Kitchener, Ontario) as described previously (*6*). The cigarettes were smoked according to either the International Organization for Standardization standard 3308 (referred to hereafter as "ISO"), with a puff volume of 35 mL, a puff duration of 2 s, and a puff interval of 60 s, or a modified smoking regime (hereafter referred to as "extreme"), with a puff volume of 70 mL, a duration of 2 s, and a 30 s puff interval.

Mainstream smoke was passed through a 92 mm glass fiber filter disk for particulate matter collection and subsequently through a cooled impinger containing phosphate-buffered saline for gas (vapor phase) collection, according to Health Canada official test methods (*24*). To collect sidestream smoke, a glass fishtail chimney was placed over the lit end of the burning cigarette, and smoke was drawn through a glass fiber filter pad. The number of cigarettes smoked for each sample and the total particulate matter (TPM) obtained are provided in Table 2.

To prepare condensate samples, the respective filter pads were placed in a flask containing dimethyl sulfoxide (DMSO) (ACS spectrophotometric grade, >99.9%) and shaken on a wrist-action

Table 2. Number of Cigarettes Smoked and the TPM Yield for Each Smoke Condensate

smoke condensate	no. of cigarettes smoked	TPM (mg)	mg TPM/ cigarette
	mainstream		
tobacco ISO[a]	20	997	50
tobacco extreme[b]	11	1249	114
marijuana ISO	13	677	52
marijuana extreme	7	910	130
	sidestream		
tobacco ISO	20	490	24
tobacco extreme	22	448	20
marijuana ISO	13	647	50
marijuana extreme	15	628	42

[a] ISO, International Organization for Standardization standard 3308. [b] Modified smoking regime designed to reflect marijuana smoking habits. See the text for details.

shaker (model 3589, Barnstead International, Melrose Park, IL) for 20 min. Each sample was standardized to a concentration of 30 mg TPM per mL of DMSO for genotoxicity testing or 10 mg TPM per mL of DMSO for cytotoxicity testing.

Neutral Red Uptake Assay. Gas and particulate phase samples of the mainstream extreme marijuana and tobacco smokes were tested for cytotoxicity using the Health Canada Official Test Method for the neutral red uptake assay (*24*). Briefly, Chinese hamster ovary (CHO) cells were seeded in 96 well plates with growth media consisting of 90% F-12, 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin and were incubated at 37 °C for 24 h. The culture medium was removed, and cells were then exposed to 0−200 μg/mL of smoke condensate for 24 h at 37 °C. Following exposure, the smoke condensates were removed and replaced with a neutral red dye, and the cells were incubated for an additional 3 h. The cells were then fixed, the dye was extracted, and the absorbance of the dye was measured at 540 nm on a microplate reader (model Elx800, Bio-Tek Instruments Inc., Winooski, VT). The relative absorbance was calculated by dividing the blank-corrected test values by the blank-corrected negative control value and multiplying by 100. The results obtained are expressed as the concentration of smoke condensate that yields a 50% reduction in dye uptake (i.e., the IC_{50}).

***Salmonella* Mutagenicity Test.** Particulate phase smoke samples were tested for mutagenic activity using the preincubation version of the *Salmonella* Mutagenicity Test as described in Mortelmans and Zeiger (*25*). Briefly, smoke condensates were combined with the *Salmonella* tester strain, a metabolic activation mixture derived from Aroclor 1254-induced rat liver (as required), and incubated for 20 min at 37 °C. The contents were then mixed with molten agar and poured onto glucose minimal media agar plates. Plates were inverted and incubated at 37 °C for 72 h. Following incubation, the number of revertant colonies on each plate was scored using a Protocol RGB Colony Counter (Synbiosis, Frederick, MD). Six bacterial test strains were used including the standard tester strains TA98, TA100, and TA102 (Moltox Inc., Boone, NC). In addition, this study also used metabolically enhanced versions of TA98 and TA100 (i.e., YG1041, YG5161, and YG1042). YG1041 and YG1042 overexpress the *Salmonella* classical nitroreductase and O-acetyl transferase enzymes and show enhanced sensitivity to nitroarenes, aromatic amines (*26*). YG5161 overexpresses the *dinB* gene encoding *Escherichia coli* DNA polymerase IV and shows enhanced sensitivity to unsubstituted PAHs (*27*). Strains YG1041, YG5161, and YG1042 were obtained directly from Dr. Takehiko Nohmi (National Institute of Health Sciences, Tokyo, Japan). Samples were tested both with and without a metabolic activation mixture. The S9 metabolic activation mixture consisted of 2 mL (2% v/v) of microsomal salt solution [0.4 M magnesium chloride and 1.65 M potassium chloride (Sigma-Aldrich Canada Ltd., Oakville, Canada)], 141 mg (4.1M) of glucose-6-phosphate (monosodium salt, Sigma-Aldrich Canada Ltd.), 306 mg (3.9M) of nicotinamide adenine dinucleotide phosphate disodium salt (NADP, Roche Diagnostics, Laval, Canada), 50 mL (50% v/v) of 0.2 M phosphate buffer, pH 7.4, 43 mL (43% v/v) of water, and 5 mL (5% v/v) of Aroclor 1254-induced rat liver S9 (Moltox Inc.). Protein levels were 35.7− 43.5 mg mL^{-1} per 100 mL of mixture, resulting in 0.9−1.1 mg of S9 protein per plate.

Cytokinesis Block Micronucleus Assay. The cytokinesis block micronucleus assay (*28*) was optimized for use with a pulmonary epithelial cell line, designated FE1, derived from the transgenic MutaMouse (*29*). Cells were seeded at a density of 5×10^5 cells/plate and cultured in DMEM F-12 supplemented with 2% v/v fetal bovine serum, 1% v/v penicillin/streptomycin, and 0.02% v/v murine epithelial growth factor (Invitrogen, Burlington, ON, Canada). Cells were grown overnight at 37 °C in a 5% CO_2 atmosphere. The following morning, cells were exposed to the particulate phase smoke condensates in serum-free media for a 4 h period and incubated for a further 28 h in the presence of 3 μg/mL cytochalasin B. Fifty microliters of DMSO was employed as a negative control (solvent blank). A 0.3 μg/mL amount of mitomycin C was used as a positive control. Cells were removed from the growth surface, pelleted, and resuspended in a 75 mM hypotonic KCl solution. Samples were fixed in 5:1 methanol:glacial acetic acid, and the cell suspensions were dropped onto ice-cold slides, washed with fixative, and dried overnight. Slides were stained with acridine orange for microscopic examination. One thousand binucleated cells were scored from each of two replicate cultures for each treatment. The frequency of cells with one, two, three, or four nuclei was determined and employed to calculate a proliferation index (PI). The PI and binucleate frequency are both useful for comparing the cytostatic effects of the test articles examined. All scoring followed the recommendations and criteria outlined by Fenech (*28*). FE1 cells have been previously shown to be metabolically competent (e.g., express cytochrome P450 1A1 and activate benzo[*a*]pyrene,) (*29*), and the cells were exposed in the absence of an exogenous activation mixture. However, because the cells may possess a limited metabolic capacity for certain substances, a smaller substudy was conducted using exogenous S9 derived from Aroclor 1254-induced rat liver. A continuous treatment protocol was also included to confirm negative responses.

Statistical Analyses. For the neutral red assay, three independent replicate assays (each consisting of four assay plates) were carried out for each tobacco and marijuana smoke sample. Because the assay results were replicated using independently generated TPM samples, analysis of variance (ANOVA) was applied to examine statistical differences in the cytotoxicity assessment values (i.e., IC_{50} in mg TPM/mL). The Student−Newman−Keuls multiple comparison procedure was used for posthoc comparison of mean values.

For the mutagenicity assay, responses were considered to be positive if a concentration-related increase in the number of revertants was observed, and the number of revertant colonies was at least double the background for at least two consecutive test concentrations. The mutagenic potency (i.e., slope) of each sample was calculated from the initial linear portion of the concentration−response function using least-squares linear regression analyses.

For the micronucleus assay results, a poisson regression model was employed to investigate the statistical significance of the concentration effect. Initial analyses determined that there was no exposure day effect and that controls were functioning properly (i.e., blanks and solvent controls not statistically different and positive controls statistically different from both blanks and solvent controls). Subsequently, the data were fitted to a model with concentration and scorer as the main effects, and the solvent exposed samples as the controls. Model adequacy was assessed by examining the deviance and plotting the predicted values against observed values. If the main effect was significant ($p < 0.05$), then contrasts for all pairwise comparisons between concentrations were compared using a Bonferroni-corrected α level that ensured an overall type I error rate of 0.05.

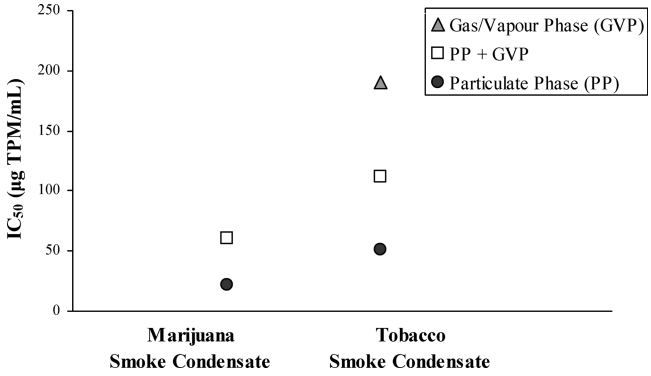

Figure 1. Mean IC_{50} values (50% inhibitory concentrations) of mainstream marijuana and tobacco smoke condensates collected under extreme conditions.

Results

Neutral Red Uptake Assay. The relative cytotoxicities of the extreme marijuana and tobacco smoke samples are shown in Figure 1. The results show that the marijuana particulate phase and combined particulate and gas phase samples (representative of whole smoke) were significantly more cytotoxic than the matched tobacco samples. However, the gas phase of the marijuana samples appears to be less cytotoxic than the matched tobacco samples, and no inhibitory concentration could be determined for marijuana at the concentrations tested. For both the tobacco and the marijuana samples, the particulate phase was substantially more cytotoxic than the combined particulate and gas phase.

***Salmonella* Mutagenicity Test.** Condensates from the particulate phase of the smoke samples were tested for mutagenicity using five different *Salmonella* strains, both with and without exogenous metabolic activation. All of the smoke condensates showed significant positive responses when tested on the strains that detect frameshift mutations (i.e., TA98, YG5161, and YG1041) in the presence of metabolic activation (Table 3). The mutagenic potencies obtained with YG1041 paralleled those of TA98 but were on average 4.5-fold higher with YG1041. A few samples elicited a positive response on the metabolically enhanced base pair mutation detecting strain YG1042, and no positive responses were elicited on strains TA100 or TA102 with metabolic activation. Although the sidestream tobacco samples elicited a positive response with some of the *Salmonella* strains, none of the marijuana samples elicited a positive response when metabolic activation was omitted (data not shown). The mutagenic potencies of each of the marijuana and tobacco smoke condensates are shown in Figure 2. Overall, the mutagenicity analyses revealed that the marijuana smoke condensates were consistently more mutagenic (1.2−7.5-fold) than the matched tobacco smoke condensates. In addition, the sidestream smoke condensates were generally more mutagenic (1.1−5-fold) than the matched mainstream condensates. The extreme smoking condition did not generate substantially greater mutagenic activity when compared to the ISO smoking regime.

Cytokinesis Block Micronucleus Assay. Range-finding studies for the cytokinesis block micronucleus assay, and concomitant calculation of the PIs, revealed that the marijuana samples are far more cytotoxic and cytostatic than the matched tobacco samples (data not shown). Consequently, the concentrations of marijuana selected for testing were approximately six times lower than the tobacco concentrations. In addition, all condensates produced under the extreme smoking condition were more cytotoxic and cytostatic than matched condensates

Table 3. *Salmonella* Mutagenicity of Tobacco and Marijuana Smoke Condensates in the Presence of S9[a]

concn	revertants/plate[b]					
(µg TPM/plate)	TA98	YG5161	YG1041	TA100	YG1042	TA102
tobacco mainstream ISO						
0	44	43	32	154	147	248
50	52	61	51	NT	226	219
100	57	88	100	165	218	245
150	72	99	141	175	213	252
250	88	135	211	164	225	256
500	126	150	260	184	211	280
800				222		
tobacco mainstream extreme						
0	44	43	32	154	147	248
50	64	64	52	NT	205	243
100	71	79	86	158	216	253
150	72	97	106	171	194	257
250	97	123	178	177	230	241
500	116	168	262	169	212	294
800				193		
tobacco sidestream ISO						
0	44	43	32	154	147	248
50	76	104	126	NT	287	236
100	99	158	232	199	273	270
150	124	179	342	207	327	265
250	150	258	419	194	401	276
500	180	289	466	233	367	290
800				277		
tobacco sidestream extreme						
0	44	43	32	154	147	248
50	69	115	154	NT	246	255
100	105	164	257	177	286	272
150	117	206	388	188	293	289
250	145	239	515	211	353	276
500	175	311	690	210	345	295
800				252		
marijuana mainstream ISO						
0	44	43	32	154	147	248
50	83	90	159	NT	257	185
100	137	161	361	187	303	203
150	195	218	545	175	325	189
250	269	366	980	195	388	205
500	462	598	1665	233	365	200
800				309		
marijuana mainstream extreme						
0	44	43	32	154	147	248
50	98	148	172	NT	289	195
100	169	216	382	197	331	197
150	205	269	566	189	404	212
250	281	347	903	209	374	213
500	328	476	1434	219	411	228
800				235	443	
marijuana sidestream ISO						
0	44	43	32	154	147	248
50	98	128	274	NT	233	214
100	145	180	452	172	291	211
150	178	231	621	170	347	204
250	246	268	1021	195	409	188
500	272	333	1221	218	438	199
800				301		
marijuana sidestream extreme						
0	44	43	32	154	147	248
50	98	99	259	NT	308	241
100	136	173	525	195	401	248
150	187	247	745	198	433	224
250	263	344	940	197	228	228
500	338	536	1500	229	236	213
800				282		

[a] NT, not tested. [b] The number of revertants per plate produced by 0.5 µg/plate of the positive control 2-aminoanthracene was 285 ± 18, 947 ± 47, 827 ± 10, 514 ± 25, and 486 ± 26 for TA98, YG5161, YG1041, TA100, and YG1042, respectively. Five µg/plate induced 863 ± 48 on strain TA102.

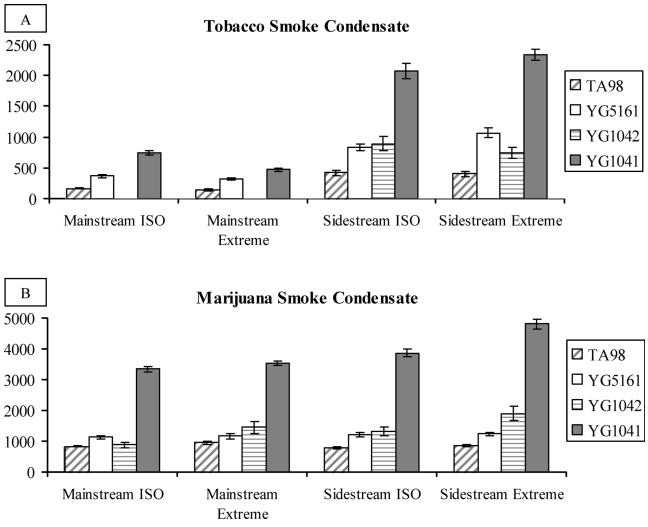

Figure 2. *Salmonella* mutagenic potency (revertants/mg TPM ± standard error) of tobacco (A) and marijuana (B) smoke condensates in the presence of an exogenous (S9) metabolic activation mixture. Note the difference in scale between plots A and B.

produced using the ISO smoking regime. As a result, these samples were also tested at lower concentrations.

Despite the aforementioned cytotoxicity, high concentrations of condensates (i.e., those eliciting 50−70% cytotoxicity assessed by PI) were examined to avoid false negative results. However, at the concentrations tested, no significant increase in micronucleus frequency was observed in cells exposed to any of the marijuana condensates (Figure 3A,B). In contrast, significant increases in micronuclei were observed for cells exposed to mainstream ISO tobacco condensates at the highest concentration (Figure 3C). However, no statistically significant increases in micronucleus frequency were observed in cells exposed to any of the other tobacco condensates (i.e., mainstream extreme, sidestream ISO, and sidestream extreme) (Figure 3C,D). For comparison, the marijuana and tobacco mainstream condensates were subsequently retested in comparison with two other archived tobacco smoke condensates (i.e., condensate from commercial brands Player's Plain and Player's Special Blend). Again, a concentration−response trend was observed for the tobacco condensates (Figure 4) but not for the marijuana (data not shown).

Because MutaMouse FE1 cells likely possess a restricted metabolic capacity, a small substudy was conducted to examine the effect of exogenous S9 activation on the ability to induce micronucleus formation. At the concentrations tested, the mainstream and sidestream ISO tobacco condensates showed a clear concentration pattern (Figure 5A), although it was not statistically significant. In contrast, the marijuana mainstream and sidestream condensates produced under the ISO smoking regime showed no clear induction of micronuclei (Figure 5B).

Discussion

For both the tobacco and the marijuana samples, the particulate phase was substantially more cytotoxic than the gas phase. Using the same assay system (i.e., neutral red with CHO cells), Rickert et al. also found that the particulate phase of mainstream tobacco smoke was more cytotoxic than the gas vapor phase (*30*). Interestingly, Tewes et al. employed the neutral red assay end point with BALB/c 3T3 cells and found opposite results; that is, the gas vapor phase was more cytotoxic than the particulate phase of mainstream cigarette smoke (*31*). Thus, although there is evidence to support the increased relative

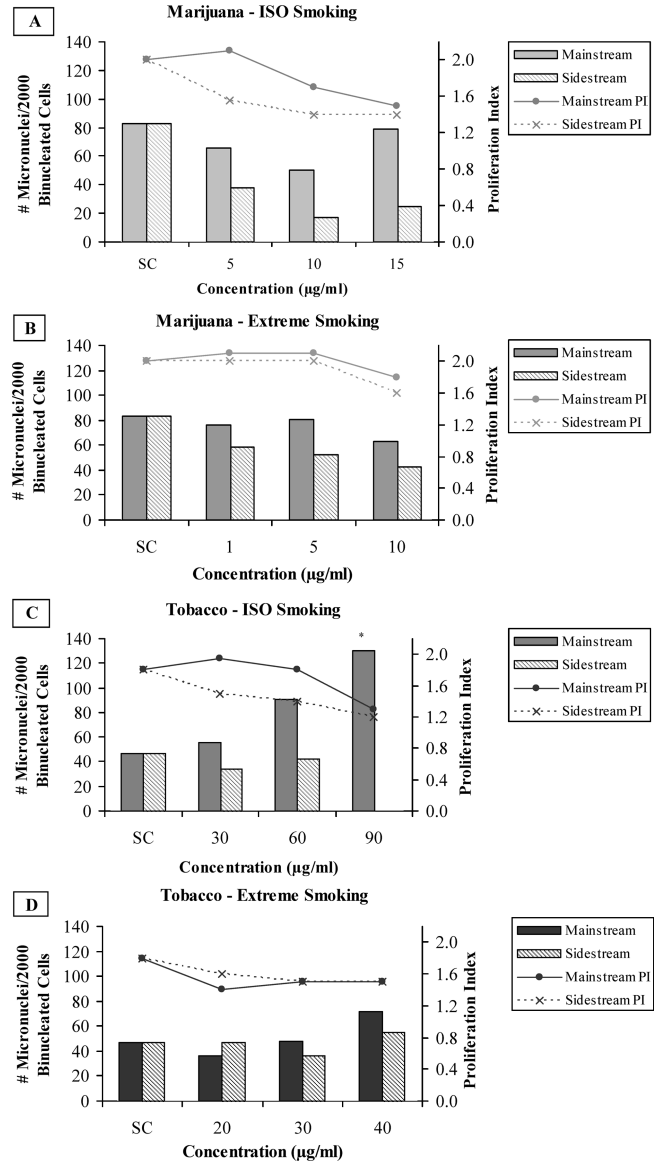

Figure 3. Micronucleus induction in FE1 cells treated with marijuana (A and B) and tobacco (C and D) smoke condensates under ISO (A and C) and extreme (B and D) smoking regimes. SC, solvent control; *significant at $p < 0.05$. Concentration units are μg TPM per assay mL.

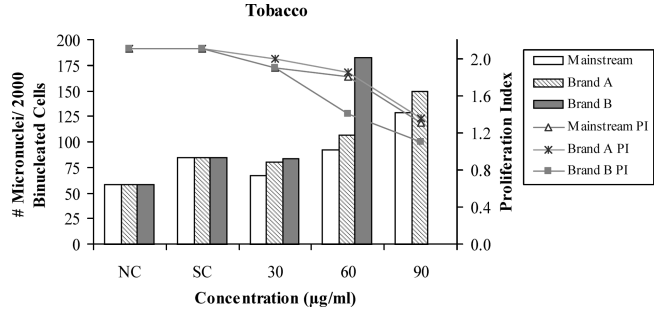

Figure 4. Micronucleus induction in FE1 cells treated with the mainstream tobacco smoke condensates used in this study, and archived tobacco smoke condensates collected under the standard ISO smoking regime. NC, negative control; and SC, solvent control.

toxicity of the particulate phase, the published research is contradictory. In addition, it is interesting to note that the experimental methods employed to assess the toxicity of the vapor phase (i.e., bubbling through phosphate-buffered saline) is somewhat analogous to water pipe smoking. Although it is

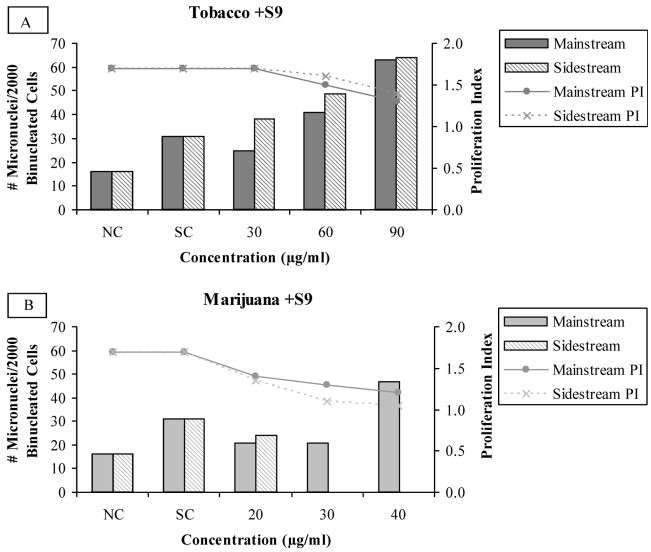

Figure 5. Micronucleus induction in FE1 cells treated with tobacco (A) and marijuana (B) smoke condensates (ISO smoking regime) in the presence of S9 metabolic activation. NC, negative control; and SC, solvent control.

unclear whether water pipe smoking can modify the toxic hazard of tobacco or marijuana smoke, the increasing use of water pipes is noteworthy (*32*), and the ability of water pipes to modify smoke toxicity is a research topic worthy of additional attention.

The results of the neutral red assay and the cytokinesis block micronucleus assay show that the marijuana particulate phase smoke condensates are more cytotoxic than matched tobacco samples. Previous studies indicate that acrolein, formaldehyde, hydrogen cyanide, and cyanohydrins (produced from the reaction of hydrogen cyanide with aldehydes) are largely responsible for the cytotoxicity of cigarette smoke (*30, 31*). A chemical evaluation of the condensates in our earlier, companion study showed that although the marijuana samples contained up to 4.6 times as much hydrogen cyanide as the matched tobacco samples, the marijuana samples contained less acrolein and formaldehyde (up to 3.7- and 9.3-fold less, respectively) than the matched tobacco samples when adjusted for TPM (*6*). Although it is likely that these chemicals contributed to the cytotoxic activity of the samples, at present time, the identity of the substances responsible for the observed results remains largely unidentified, and it is not clear how the relative concentrations of the highlighted chemicals will affect toxicity.

The *Salmonella* mutagenicity analyses show that the marijuana smoke condensates are consistently more mutagenic than the matched tobacco samples for both mainstream and sidestream smoke, under both ISO and extreme smoking regimes. In previous studies, Wehner et al. (*2*) also found mainstream marijuana condensates to be more *Salmonella* mutagenic than two commercial brands of tobacco cigarettes. However, Busch et al. (*10*) noted that the mutagenic activity of marijuana smoke condensates are comparable to, but not more mutagenic than, tobacco condensates. The discrepancy between these two studies may be attributed to differences in the types of tobacco and marijuana analyzed. The mutagenic potency of different tobacco cigarettes has been noted, and the differences have been attributed to variations in the source of the tobacco leaves, the type of tobacco blend, the nature of the processing, and the cigarette construction (*33*−*36*). In addition, these earlier studies did not examine marijuana and tobacco cigarettes prepared and combusted under matched conditions (e.g., same rolling paper, cigarette weight, and smoking regime). Therefore, direct comparisons between the mutagenic potencies of the tobacco and marijuana smoke condensates were likely confounded by these differences.

Early studies suggested that higher levels of PAHs found in marijuana samples were likely responsible for the higher mutagenic activities (*37, 38*). However, our earlier study revealed that mutagenic PAHs were rarely present in greater concentrations in the marijuana condensates (*6*). Indeed, with the exception of dibenz[*a*,*h*]anthracene in mainstream smoke and naphthalene in sidestream smoke, TPM-corrected PAH concentrations were consistently lower concentrations in the marijuana condensates. In addition, an analysis of the mutagenic potencies of the smoke condensates that would be predicted from the observed concentrations of mutagenic PAHs showed that these compounds can only account for less than 0.005% of the mutagenic activity of marijuana smoke and less than 0.05% of the mutagenic activity of the tobacco smoke (Table 4). Other studies have found similar results with PAHs accounting for 0.03% of the TA98 mutagenic activity of Kentucky Reference cigarettes 1R4F (*31*), and it is generally agreed that PAHs alone cannot account for the observed mutagenic activities of cigarette smoke condensate (*10, 39*−*41*).

More recent work has suggested that aromatic amines and heterocyclic amines are responsible for a substantial portion of the *Salmonella* mutagenicity of smoke condensates (*42, 43*). Many of these compounds are produced during the pyrolysis of amino acids and proteins at high temperatures, and studies have confirmed that the removal of these proteins prior to tobacco combustion results in substantially reduced mutagenic activities (*44*). Although our earlier, companion study examined only four aromatic amines (i.e., 1- and 2-aminonaphthalene and 3- and 4-aminobiphenyl), each of these compounds were present in notably higher concentrations in the mainstream marijuana condensates than in the matched tobacco condensates (*6*). Moreover, the enhanced mutagenicity observed on the metabolically enhanced strain YG1041 (with S9) for both tobacco and marijuana suggests an important contribution from mutagenic aromatic amines. Thus, a substantial portion of the mutagenic activity of the marijuana smoke condensate appears to be due to heretofore unidentified aromatic amines and/or N-heterocyclic compounds.

In this study, both the tobacco and the marijuana condensates were more mutagenic on strains that detect frameshift mutagens (i.e., TA98, YG1041, and YG5161) in comparison with strains that detect base pair mutagens (i.e., TA100 and YG1042). These findings are consistent with previous studies (*2, 10, 40*) that show a predominance of frameshift mutagenicity for both marijuana and tobacco smoke condensates.

The sidestream smoke condensates were generally observed to be more mutagenic than the matched mainstream condensates for both marijuana and tobacco. Although the chemical constituents are qualitatively similar, differences in pH and combustion temperature have been shown to contribute to quantitative differences in the chemical composition of mainstream and sidestream smoke (*45*). Indeed, sidestream tobacco smoke has been previously found to contain higher concentrations of carcinogens than mainstream smoke (*46, 47*). The presence of higher concentrations of selected chemicals in sidestream marijuana and tobacco smoke, including some mutagens and carcinogens (e.g., PAHs, aromatic amines, aldehydes, and NOx), in comparison with mainstream smoke condensates, was confirmed in our earlier study (*6*).

Although smoke contains numerous carcinogens and mutagens, there are also many cytotoxins, mutagens, free radicals,

Table 4. Predicted *Salmonella* TA98 Mutagenic Potency of Mainstream Smoke Condensates Based on the Potency[a] and Concentration of Selected PAHs

PAH	marijuana mainstream ISO		tobacco mainstream ISO		marijuana mainstream extreme		tobacco mainstream extreme	
	concn (ng/mg TPM)	predicted contribution (rev/mg TPM)	concn (ng/mg TPM)	predicted contribution (rev/mg TPM)	concn (ng/mg TPM)	predicted contribution (rev/mg TPM)	concn (ng/mg TPM)	predicted contribution (rev/mg TPM)
benz[*a*]anthracene	0.50	2.4×10^{-3}	0.61	2.9×10^{-3}	0.33	1.6×10^{-3}	0.46	2.2×10^{-3}
benzo[*b*]fluoranthene	0.14	2.6×10^{-4}	0.22	4.2×10^{-4}	0.12	2.4×10^{-4}	0.19	3.7×10^{-4}
benzo[*k*]fluoranthene	0.03	8.4×10^{-5}	0.07	2.0×10^{-4}	0.03	1.0×10^{-4}	0.06	1.9×10^{-4}
benzo[*a*]pyrene	0.17	4.1×10^{-2}	0.29	7.1×10^{-2}	0.12	2.9×10^{-2}	0.22	5.4×10^{-2}
indeno[1,2,3,-*cd*]pyrene	0.07	8.9×10^{-4}	0.09	1.2×10^{-3}	0.07	3.6×10^{-4}	0.09	1.1×10^{-3}
benzo[*g*,*h*,*i*]perylene	0.05	3.8×10^{-4}	0.08	5.9×10^{-4}	0.05	8.5×10^{-4}	0.06	4.9×10^{-4}
total	0.96	4.5×10^{-2}	1.35	7.6×10^{-2}	0.72	3.2×10^{-2}	1.09	5.9×10^{-2}
actual mutagenic potency (rev/mg TPM)	837		167		950		136	
% contribution of PAHs to overall mutagenic potency	0.005		0.045		0.003		0.043	

[a] *Salmonella* TA98 mutagenic potency values for these PAHs are as follows: 4.71, 9.65, 2.88, 245.85, 12.80, and 7.75 revertants/μg PAH, respectively (Gagnon, unpublished).

tumor promoters, cocarcinogens, and compounds that cause cancer through epigenetic mechanisms and compounds that have yet to be classified as carcinogens present in the smoke condensates (*48*). Thus, it was of interest to investigate potential empirical relationships between concentrations of chemical analytes as determined in our earlier companion study and the bioassay responses (e.g., mutagenic potency) determined in the current study. Although a variety of statistical techniques were employed, we were unable to identify statistically robust relationships due to the lack of toxicological data (four marijuana observations and four tobacco observations). Therefore, while a relationship between the biological data and one or several of the chemical analytes may exist, the current data were not adequate to explore this hypothesis. Additional studies with more replicates within each combination of sample type (i.e., marijuana or tobacco), stream (i.e., mainstream or sidestream), and smoking regime (i.e., ISO or extreme) would be required to thoroughly investigate these empirical relationships.

The chemical composition of smoke is also known to be influenced by the smoking regime (*46*). Our earlier study noted that mainstream samples smoked under the extreme regime contained almost 2.5 times as much TPM in comparison with the samples smoked under the ISO regime. Therefore, although notable differences in chemical concentrations were initially observed between samples smoked under the ISO and extreme conditions, TPM-corrected values showed similar concentrations of chemical analytes. In general, the mainstream extreme samples contained either equal or slightly lower concentrations of chemicals per unit TPM than samples smoked under the ISO conditions. These findings are consistent with the mutagenicity results that showed slightly less mutagenic activity for the extreme tobacco samples in comparison with the ISO samples. Although the marijuana mainstream extreme samples tended to be more mutagenic than the ISO samples, the results were comparable. More specifically, the results for TA98, YG1041, and YG5161 showed relatively small increases in mutagenic potency of 13.6, 5.6, and 3.7%, respectively. The results for YG1042 showed a 64% increase in mutagenic potency for the extreme sample. Similar findings have been noted in other studies where extreme smoking methods result in higher TPM yields and reduced mutagenic activity expressed per unit TPM (*30, 49*).

Although the marijuana condensates were mutagenic in the *Salmonella* mutagenicity assay, they did not elicit significant positive responses in the cytokinesis block micronucleus assay. The cell culture period was optimized to avoid missing a clastogenic event, and chronic studies, although not exhaustively investigated, also failed to reveal micronucleus formation (data not shown). Because marijuana smoke has been shown to be genotoxic in other mammalian cell assays (*11*−*13*), it seems possible that the enhanced acute toxicity of the marijuana condensates examined in this study, as compared to the tobacco condensates, prevented the manifestation of micronucleus induction. Alternatively, it is possible that the cannabinoid THC in the marijuana condensates provided protection against clastogenic effects. THC has been shown to both induce and subsequently competitively inhibit the enzyme cytochrome P450 1A1, the enzyme responsible for metabolizing and activating many carcinogenic PAHs and heterocyclic amines (*50*). Protection against mutagenic effects has been previously noted for a variety of ingested phytochemicals (*51*). These findings appear to be consistent with the limited number of existing publications that show a correlation between marijuana smoke exposure and pulmonary toxicity but no correlation with respiratory cancers (*17*).

In contrast to the marijuana condensates, the mainstream ISO tobacco condensate induced a significant increase in micronuclei frequency at the highest concentration tested. Although no statistically significant differences were identified, concentration−response trends were also observed when archived mainstream ISO tobacco smoke condensates were tested for micronucleus induction. Previous studies that exposed Chinese hamster lung cells to tobacco condensates (*52*) and whole smoke (*53*) also noted a significant increases in micronucleus formation. More recently, a study exposing L5178Y/*Tk*± 7.3.2C mouse lymphoma cells to 10 different cigarette smoke condensates in the presence of S9 noted micronuclei induction for all condensates (*54*). However, none of the other tobacco samples (i.e., extreme or sidestream) elicited a significant positive response on the CBMN assay. Again, it is suspected that the acute toxicity of these samples prohibited recovery and observation of micronucleated cells. This would not be unexpected given that the extreme and sidestream condensates were all more cytotoxic/cytostatic than the matched mainstream ISO condensates.

Although FE1 cells have been previously shown to express cytochrome P450 1A1 and activate benzo[*a*]pyrene (*29*), a smaller substudy was conducted to examine the ability of marijuana and tobacco mainstream and sidestream ISO condensates to induce micronucleus formation in the presence of exogenous metabolic activation. The results of this substudy paralleled the results obtained without S9. That is, at the concentrations tested, the marijuana condensates did not elicit an increase in the frequency of micronucleus induction, whereas the tobacco condensates

Table 5. Summary of Cytotoxicity, Mutagenicity, and Micronucleus Results for Tobacco and Marijuana Smoke Condensates[a]

	tobacco				marijuana			
	mainstream ISO	mainstream extreme	sidestream ISO	sidestream extreme	mainstream ISO	mainstream extreme	sidestream ISO	sidestream extreme
neutral red −S9	NT	50.8[b]	NT	NT	NT	22.3[b]	NT	NT
				Salmonella mutagenicity +S9				
TA98	+	+	+	+	+	+	+	+
YG5161	+	+	+	+	+	+	+	+
YG1041	+	+	+	+	+	+	+	+
TA100	−	−	−	−	−	−	−	−
YG1042	−	−	+	+	+	+	+	+
TA102	−	−	−	−	−	−	−	−
				Salmonella mutagenicity −S9				
TA98	−	−	+	−	−	−	−	−
YG5161	−	−	+	+	−	−	−	−
YG1041	−	−	+	+	−	−	−	−
TA100	−	−	−	+	−	−	−	−
YG1042	−	−	−	−	−	−	−	−
TA102	−	−	−	−	−	−	−	−
micronuclei +S9	concn response	NT	concn response	NT	−	NT	−	NT
micronuclei −S9	significant concn response	concn response	−	−	−	−	−	−

[a] NT, not tested; −, negative according to the assay standards; and +, positive according to the assay standard. [b] Fifty percent inhibitory concentration (μg TPM/mL).

showed a clear concentration−response pattern, although the response pattern was not statistically significant.

Conclusion

This study evaluated the relative ability of matched marijuana and tobacco condensates to induce (geno)toxic responses in three in vitro test systems. The results obtained provide a comparative summary for matched sidestream and mainstream condensates as well as condensates prepared under both ISO and extreme smoking regimes (Table 5).

The results indicate that tobacco and marijuana smoke differ substantially in terms of their cytotoxicity, mutagenicity, and ability to induce chromosomal damage. Specifically, the marijuana condensates were all found to be more cytotoxic and mutagenic than the matched tobacco condensates. In contrast, the tobacco condensates induced cytogenetic damage in a concentration-dependent manner, whereas the marijuana samples did not.

Little difference was noted in the mutagenic activity per unit TPM of samples smoked under the extreme vs the ISO regime. However, on a per cigarette basis, the mutagenic activity of extreme samples was substantially higher. This study, and our earlier companion study, also showed that sidestream smoke condensates generally contain higher concentrations of chemical analytes and are more cytoxtoxic and mutagenic than matched mainstream condensates.

Although this study raises important issues regarding the relative (geno)toxic potential of marijuana smoke and the potential for induction of adverse health effects, it is important to note that the study only examined a limited number of toxicological end points. In addition, potent mutagens identified by one toxicological assay are not necessarily potent mutagens in another. This has been demonstrated in a recent study examining several types of cigarette smoke condensates under different smoking conditions that found no relation among the genotoxic potencies of the condensates across four toxicology assays employed (*54*). Therefore, while the findings of the current study could have important implications for communication strategies regarding the harm associated with marijuana use, the results have qualitative but not quantitative value in evaluating potential health risks.

It is also important to note that variations in the physical−chemical properties of tobacco and marijuana cigarettes will likely modify the relative (geno)toxic activity, and the results obtained should be confirmed. Future studies should evaluate the validity of the results obtained across a variety of marijuana products and, moreover, investigate the comparative toxicological properties of marijuana and tobacco smoke in animal models.

We are currently exploring the toxicological pathways induced by marijuana smoke exposure through the use of genome wide expression profiling. Preliminary results reveal 4700 differentially expressed genes in cells exposed to marijuana smoke condensate, in contrast to 2400 differentially expressed genes in cells exposed to tobacco smoke condensate, and an overlap of 1422 genes common to both condensates.

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