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A responsive, sensitive, and reproducible dermal tumor promotion assay for the comparative evaluation of cigarette smoke condensates

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Abstract

The mouse dermal initiation/promotion bioassay has been used for several decades to study cigarette smoke condensates (CSCs). However, these studies have used highly variable methodologies that differ in the manner of CSC collection, duration of treatment, mouse strain, number of mice and endpoints measured. In this report, a protocol that uses female SENCAR mice and standardizes many of the procedures is presented. A reference cigarette (University of Kentucky 1R4F), readily available to researchers, was used. This report presents the combined data from four independent studies. Female, SENCAR mice (40/group) were treated with a single dose (75 μg) of dimethylbenz[a]anthracene (DMBA) as an initiator, followed 1 week later by treatment (three times/week) with 10, 20 or 40 mg "tar"/application of 1R4F CSC for 29 weeks. There were no treatment-related effects on body weights. Histological diagnosis of all masses at study termination indicated a dose-dependent increase in the number of tumor-bearing mice and total tumor number. These studies support the conclusion that the 1R4F cigarette is suitable for use as a reference standard and the protocol presented is an appropriate and standardized model suitable for the comparative evaluation of CSC.

Keywords: Dermal tumor promotion; Cigarette smoke condensate; 1R4F; Comparative assay; SENCAR

1. Introduction

Cigarette design has evolved for over a century with the incorporation of many new technologies in the last several decades (Norman and Jones, 1998). Modifications to cigarette design have historically involved the incorporation of new ingredients, tobacco processes, papers, and filters that have the potential to modify the quantity and quality of smoke yielded from the cigarette (Swauger et al., 2002). Evaluation of materials such as ingredients added to tobacco, processed tobaccos, and structural materials (cigarette papers, adhesives, inks, and filter materials) used to manufacture cigarettes is an issue addressed in some tobacco-related legislation and/or regulation surfacing globally. While these regulations do address the general subject of the evaluation of such materials, they typically do not address the practical matter of how such evaluations should be conducted.

The US Food and Drug Administration has employed tiered testing strategies to evaluate direct food additives and color additives in food for approximately 20 years (USFDA, 1982, 1993). This concept is based on the assumption that the degree of effort expended to reduce the uncertainty about the safety of an additive should relate in some logical way to the actual likelihood that the additive poses a health risk to the public. Similarly, tiered testing strategies have been developed to provide a conceptual framework, based on a level of concern, which facilitates the design of toxicological evaluation programs. These programs evaluate the potential for new materials used in the manufacture of cigarettes, changes in tobacco processing, or the

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development of new technologies to increase or reduce the biological activity that results from burning tobacco (deBethizy et al., 1998; Swauger et al., 2002; R.J. Reynolds Tobacco Co., 1988a).

Depending on the specific nature of the proposed modification, these comparative toxicological evaluations could include an examination of a number of mainstream smoke constituents (which have been identified by various regulatory agencies to have the potential to contribute to the risks associated with smoking) as well as in vitro and in vivo toxicology. Appropriate toxicological models have been selected for use in these evaluations based on their relevance to specific disease states, demonstrable responsiveness to tobacco smoke or to cigarette smoke condensate (CSC), and demonstrable sensitivity sufficient to allow differentiation between either cigarette smoke or CSC of varying toxicity. At present, there are a limited number of toxicological models that meet these basic criteria.

Rodent dermal tumorigenesis models represent a significant subset of the toxicological models that have been traditionally employed to evaluate cigarettes and proposed modifications to cigarettes. Dermal tumorigenesis studies have been used to evaluate CSC since the early studies of Wynder et al. (1953) in the 1950s. These early studies reported CSC was a weak tumorigen when repeatedly applied to the shaved backs of mice for as long as 52 weeks. Van Duuren et al. (1966) reported CSC was a promoter of 7,12-dimethylbenz[a]anthracene (DMBA)-initiated tumorigenesis in a mouse dermal initiation/promotion assay. The National Cancer Institute (NCI) chose an 18-month mouse dermal tumorigenesis assay as an important component of its cigarette research program (NCI, 1980). Dermal tumorigenesis assays have been used in a number of studies evaluating fractionated CSC and tobacco extracts in attempts to determine the source of CSC activity (Whitehead and Rothwell, 1968; Bock et al., 1969; Bock, 1970; Hoffman and Wynder, 1971; Akin and Chamberlain, 1974; Lee et al., 1977; Van Duuren and Goldschmidt, 1976). These assays have also been used to investigate the activity of individual components of CSC (Lee et al., 1977) and to investigate the effect of cigarette design variables on the activity of CSC (NCI, 1980; Roe et al., 1970; Halter and Ito, 1972; Dontenwill et al., 1972; Bock et al., 1974; Bernfeld and Homburger, 1976; Dontenwill et al., 1977; Gaworski et al., 1999).

The mouse skin tumor model (MST) has been developed for use in short-term carcinogenicity testing utilizing the SENCAR mouse as the standard strain for both initiating and promoting effects (Slaga, 1986; NTP, 1996: Enzmann et al., 1998). The MST, however, has limitations. The use of a strain that is more sensitive than others may increase the chance of detecting strain-specific effects without relevance to other strains and species, including humans (Kraus et al., 1995; Enzmann et al., 1998). Although both initiation and promotion stages are monitored, the stage of progression is not and the interference of irritant activities may not be excluded. Finally, the topical route of exposure is not the major route of potential human exposure. Nevertheless, if the dose selection is appropriate, it can be a very meaningful short-term bioassay (Kraus et al., 1995: Enzmann et al., 1998).

To obtain quantitative, reproducible data from dermal initiation/promotion studies, protocol design standards need to be developed (Eastin, 1989; Schmidt and Hecker, 1989; Edler et al., 1991). A review of previous CSC studies using the mouse dermal initiation/promotion assay indicates that a number of factors are necessary to produce a reproducible, quantitative assay. These include: (1) a standardized CSC collection method, (2) a quantitative dose–response, (3) short-term results, (4) a test species and strain that is readily available and is sensitive to the materials under evaluation, and (5) the ability to periodically verify responsiveness with a reference cigarette. The protocol presented in this report meets these criteria and provides a highly reproducible, quantitative study design to assess the tumor promotion activity of CSC.

As part of product stewardship efforts, CSC from the University of Kentucky 1R4F (1R4F) reference cigarette was included in four dermal tumor promotion studies conducted between 1994 and 2000. Presentation of these individual studies would demonstrate the responsiveness of the bioassay. By combining the data of the four studies, the reproducibility of the bioassay over time can be presented and the experimental variability can be demonstrated. The combined results reported here provide quantitative data concerning the promotional activity of 1R4F CSC in the SENCAR mouse dermal tumor promotion bioassay. The presented data also demonstrate the suitability of the 1R4F cigarette as a reference standard useful in validating dermal initiation/promotion protocols and ensuring the protocols provide consistent data over time. Overall, the results presented demonstrate that this dermal tumor promotion assay is appropriate, reproducible, and suitable for the comparative evaluation of CSCs.

2. Materials and methods

2.1. Cigarette

These studies used the 1R4F reference cigarette obtained from the Tobacco and Health Research Institute, University of Kentucky (Lexington, KY). This cigarette was developed to represent an average filtered full flavor low-"tar" American cigarette. It is made available to researchers as a standard cigarette to maintain consistency among various cigarette smoke studies done in different laboratories and within the same laboratory.

It is an air-diluted, filtered cigarette containing a blend of tobaccos [flue-cured (32.5%), Burley (20.0%), Oriental (11.1%), Maryland (1.1%), and reconstituted tobacco sheet (27.2%)] with glycerol (2.8%) and added sugars (5.3%) (Sullivan, 1984). 1R4F cigarettes were stored at 4 °C until use. Cigarettes were conditioned to the laboratory environment (17.8–26.1 °C and 30–50% relative humidity) for approximately 18 h before being smoked for condensate collection.

2.2. Collection of cigarette smoke condensate

Cigarettes were smoked on an AMESA Type 1300C smoke generator (Amesa Aeromecanique, Geneva, Switzerland) within a fume hood. They were placed in a 30-port head that rotated (1 rpm) so that each cigarette was exposed to a vacuum port. The vacuum port contacted each cigarette individually and drew smoke from one cigarette at a time. Standard Federal Trade Commission (FTC) smoking conditions (35 ml puff of 2 s duration taken once/min to a butt-length of 3 mm above the filter overwrap) were used to collect smoke from each cigarette (Pillsbury et al., 1969). Cigarettes were lit automatically with a natural gas flame. Butts were automatically ejected by compressed air and cigarettes automatically replaced in the 30-port head. Vacuum was provided by a Masterflex peristaltic pump (Cole-Parmer, Chicago, IL). Silicon tubing was used between the vacuum port and the first condensate collection impinger.

Smoke from the vacuum port was delivered to a CSC cold-trapping system. Impingers, maintained in Dewar flasks containing isopropyl alcohol/frozen CO_2, were contained within a vented fume hood. The system consisted of six impingers connected in series by silicon tubing. The impingers had a 500 ml volume and were fitted with 1.5 in. of glass beads (10–14 mm dia.) overlaid with 8 in. of smaller glass beads (5–7 mm dia.). The first two impingers were maintained at a temperature of −10 °C. The next two impingers were maintained at −50 °C and the last two impingers were maintained −70 °C. Temperatures in the Dewar flasks were monitored throughout the condensate collection period via a thermocouple and the Dewar flasks maintained within ±5 °C of the intended temperature. The flow from the last impinger was exhausted. At completion of a single smoking session (approximately 7000 cigarettes), condensate was frozen at −20 °C pending extraction. Condensate was extracted from the impingers and glass beads by rinsing with high-purity acetone [“Nanograde” acetone (Mallinckrodt Chemical, Chesterfield, MO) or “Optima” acetone (Fisher Scientific, Pittsburgh, PA)]. Several daily condensate collections were combined to create a pooled CSC sample.

Acetone and water content of the pooled condensate was reduced by rotary evaporation at 40 °C and a vacuum pressure of 19 psi using a Buchi R-124 Safetyvap (Flawil, Switzerland) at 60 rpm. The water content was reduced to <8%, as suggested by the National Cancer Institute (NCI, 1980). CSC from 3–5 collections was pooled and again subjected to acetone and water removal to yield the CSC preparations from which each set of dosing solutions were prepared. In these studies, each batch of pooled CSC was subjected to gas chromatography (GC) analysis for water, nicotine, glycerol (Borgerding et al., 1990), pH, benzo(a)pyrene (Dumont et al., 1993), hydroquinone, catechol, phenol, and *p*,*m*-cresol (Risner and Cash, 1990). Selection of these constituents for analysis was based upon their use to measure condensate components and/or their selection by the National Cancer Institute (NCI, 1976). Other constituents could be determined based upon the specific experimental design. “Tar” content was determined by analysis for nicotine and water followed by subtraction of the quantities of nicotine and water from the quantity of total particulate matter (NCI, 1980). Condensate was stored in amber bottles at −20 °C for up to 7 weeks. CSC was collected 5–6 times during the course of each study.

2.3. Preparation of CSC dosing solutions

Dosing solutions were prepared every 5–6 weeks by serial dilution of aliquots of freshly collected, pooled CSC with 8% water/acetone (v/v) to maintain an 8% water content (NCI, 1980). Concentrations of the dosing solutions were verified by GC analysis for nicotine. “Tar” concentrations (mg “tar”/application) of the dosing solutions were calculated by multiplying the measured nicotine concentrations (mg/ml) by the dilution assigned to the dosing solutions, then dividing by the predicted nicotine value (mg/ml). This is based upon the constant “tar” to nicotine ratio in CSC from the same cigarette type. The mid-dose dosing solution was prepared from the high-dose dosing solution by dilution. If the mid-dose solution was within specification in respect to “tar” concentration (±10%), the low-dose solution was prepared from the mid-dose and its “tar” concentration confirmed.

Dosing solution stability was determined by periodically (at weeks 1, 2, 3, 4, 6, 8, 10, 12, and 14) determining nicotine concentration. Dosing solutions were found to be stable for at least 14 weeks using this criterion. They were stored in amber glass bottles at −20 °C for up to 7 weeks.

2.4. 7,12-Dimethylbenz[a]anthracene dosing solution

7,12-Dimethylbenz[a]anthracene (DMBA) was obtained from Aldrich Chemical Company (Milwaukee, WI) and stored according to the manufacturer’s recommendations. Purity of the DMBA was confirmed to be 98.5% by gas chromatographic/mass spectral (GC/MS) analysis. The dosing solution was prepared in high-purity

acetone and stored in an amber bottle at −20 °C. Concentration of the DMBA dosing solution was confirmed by gas chromatography using flame ionization detection after preparation (before administration to the mice) and again 1 week later (after application to the mice).

2.5. Acetone dosing solution

High-purity acetone was used as the solvent for CSC and DMBA and as the vehicle control in these studies. Acetone purity was confirmed to be $\geqslant 99.6\%$ by GC/MS prior to dosing.

2.6. Animals

Female, SENCAR mice, age 4–6 weeks, were obtained from Harlan Sprague–Dawley (Indianapolis, IN) or the National Cancer Institute, Frederick Cancer Research and Development Center, Animal Program (Frederick, MD). SENCAR mice were selected because of their high sensitivity in dermal initiation/promotion assays (Slaga, 1986; Robinson, 1986). A substantial database has been developed for this strain regarding in-life variables (growth rate and longevity) and in the background incidence of spontaneous pathological lesions (Knutsen et al., 1986; Kovatch et al., 1986; Ward et al., 1986). They have also been used to assess the carcinogenic potency of complex mixtures including diesel and gas engine emissions (Slaga and Triplett, 1981).

Within the first week after arrival, 20 mice from each study were subjected to serologic evaluation [pneumonia virus of mice, Sendai, mouse hepatitis virus, minute virus of mice, mouse polio virus (GDV11), Reo type 3 virus, Polyoma virus, Ectromelia virus, mouse pneumonitis virus, mouse adenovirus, epizootic diarrhea of infant mice, mouse cytomegalovirus, lymphocytic choriomeningitis virus, *Mycoplasma pulmonis*, Hantaan virus, *Encephalitozoon cuniculi*, mouse thymic virus, and cilia associated respiratory bacillus (Charles River Laboratories, Wilmington, MA)] and necropsied for signs of ill health. Lungs were examined microscopically after fixation, sectioning, and staining with hematoxylin/eosin to ascertain health status. Study initiation depended upon negative serology and release of the mice to the study by the attending veterinarian. An additional 20 sentinel mice from each study were subjected to serologic evaluation and necropsy at study termination to determine colony health. No significant lesions, pathogens or viral antibodies were detected at the conclusion of three of the four studies. After initiation of one study, the animal supplier reported positive titers for mouse orphan parvovirus in their colony. Our mice also tested positive but there was no indication that this infection altered the outcome or conclusions from that study.

Mice were housed and cared for according to the Animal Welfare Act of 1970 and amendments (Public Law (1-579), as set forth in CFR Title 9, Part 3 Subpart F). Prior to conduct of the studies, protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Mice were individually housed and identified by cage card during the 10- to 14-day quarantine period. They were allocated to study groups by body weight using Xybion PATH/TOX software (Version 4.1.7) (Xybion Medical Systems, Cedar Knolls, NJ). Body weights were compared by ANOVA and least significant difference criteria to insure group mean body weight did not differ at a 5% two-sided probability level. After allocation, mice were uniquely identified by tail tattoo.

Mice were individually housed in suspended, stainless steel, wire-bottomed cages without bedding. Caging was changed each week. The location of each treatment group within a caging rack was rotated every other week in a pattern to ensure a balanced contribution to the study variance. In addition, the location of each caging rack within the room was rotated every other week. Animal room conditions were set to maintain a temperature of 22 ± 5 °C and relative humidity of 30–70% and continuously recorded. Filtered (HEPA and charcoal) air was provided to the room at a rate to ensure a minimum of 10 air changes per hour. Light from low UV fluorescent lamps was on a 12-h light/dark cycle with lights on at 06:00. Mice were fed pelleted Certified Purina Rodent Chow 5002 (PMI Feeds, St. Louis, MO) ad libitum. Distilled water was provided ad libitum to the mice via an automatic watering system.

2.7. Tumor promotion assay

The basic study design is presented in Table 1. Mice were initiated with a single 200 μl application of acetone (Groups 1 and 6) or a single 200 μl application of 75 μg DMBA in acetone (Groups 2–5). One week later, promotion was begun with 200 μl applications of acetone (Groups 1 and 2) or CSC (Groups 3–6) three times per week (Mondays, Wednesdays, and Fridays), which continued for 29 weeks.

CSC doses of 0, 10, 20, and 40 mg "tar"/dosing day were chosen to avoid unacceptable toxicity but provide acceptable tumor promotion. [Although not observed in the studies reported here, at times, mice treated with the high-dose "tar" (40 mg "tar"/200 μl acetone) may exhibit signs of acute toxicity during the first few weeks, probably related to nicotine. When acute toxicity is evident, treatment can be changed to 50% of the intended doses in 100 μl acetone applied twice each treatment day. When signs of acute toxicity diminish, generally after 3–4 weeks, these groups can be returned to a single 200 μl administration per treatment day.] The DMBA/acetone control serves to account for any non-CSC promoted tumors. CSC is a weak chemical initiator and a moderate tumor promoter. The acetone/CSC treated

Table 1
Study design

Group	Treatment		Number of mice	Length of treatment		Frequency of treatment	
	Initiation (dose)	Promotion[a] (dose)		Initiation	Promotion	Initiation	Promotion
1	Acetone (200 μl)	Acetone (200 μl)	40	1 Day	29 Weeks	1	3/week
2	DMBA[b] (75 μg)	Acetone (200 μl)	40	1 Day	29 Weeks	1	3/week
3	DMBA (75 μg)	1R4F CSC[b] (10 mg/day)	40	1 Day	29 Weeks	1	3/week
4	DMBA (75 μg)	1R4F CSC (20 mg/day)	40	1 Day	29 Weeks	1	3/week
5	DMBA (75 μg)	1R4F CSC (40 mg/day)	40	1 Day	29 Weeks	1	3/week
6	Acetone (200 μl)	1R4F CSC (40 mg/day)	40	1 Day	29 Weeks	1	3/week

[a] Promotion was started 7 days after initiation.
[b] Dose volume was 200 μl for DMBA and CSC dosing solutions.

group, when compared to the acetone/acetone control, serves to evaluate complete carcinogenic potential within the context of this experimental design (i.e., 29 weeks of exposure).

Once per week (on a non-dosing day), electric clippers were used to remove the hair from each mouse from the nape of the neck to the base of the tail and laterally to shoulders and hips. Dose administration was by application to the mid-back of the mice using a mechanical pipette equipped with disposable tips. The dose was carefully spread over the clipped area with the pipette tip. The pipette tip was changed between each treatment group.

2.8. In-life data

Moribundity and mortality observations were conducted twice daily, Monday through Friday. On weekends and holidays, observations were conducted once a day. Moribund mice were euthanized and necropsied and the tissues maintained in buffered 10% formalin for histopathological evaluation. Detailed clinical observations were conducted weekly. Body weights were recorded on the day of delivery, at allocation to study groups, then weekly for 10 weeks, at week 12, and then at 4-week intervals.

Dosed areas were carefully observed weekly for desquamation, discoloration, edema, erythema, eschar, fissuring, ulceration, clipper nicks, masses, and other abnormalities. A mass was defined as a circumscribed growth or swelling arising from the skin. Mass data were recorded by location, length, width, and height on individual ''mass maps'' that consisted of computer diagrams in the Xybion system. Subjective diagnosis of masses was not attempted during the in-life phase of these studies because all masses were subjected to histological diagnosis at study termination.

2.9. Necropsy

All surviving mice were weighed and subjected to complete necropsy at study termination. Final ''mass maps'' were verified, including notation of all observed masses with their descriptions. The dorsal skin from the application site was removed and its orientation denoted by leaving the ears attached. A corresponding sample of inguinal skin was removed. Excised skin was placed on index cards and immersed in buffered 10% formalin fixative to insure it was fixed in a flat position.

Approximately 40 other tissues were removed and preserved in fixative for potential histological examination. Brain, heart, liver, lungs, kidneys, spleen, adrenal glands, ovaries, and any gross lesions from the high-dose and control groups were subjected to histopathological examination. A 0–5 (normal–severe) grading scale was used in evaluation of the histopathology. Histopathological data were entered into the Xybion system. Skin masses, skin lesions (i.e., irritation, desquamation, sores, etc.), dosed skin exhibiting no apparent masses or lesions, and undosed skin from the inguinal region from all study groups (except sentinel mice) were designated for histopathological evaluation after eosin/hematoxylin staining. The histologic sections of skin masses were evaluated only for tumor type, number, and factors associated with whether the tumor was benign or malignant. Non-neoplastic changes in the skin surrounding a mass were not diagnosed or quantified because these changes were not associated with treatment of the skin in any defined manner. Changes in this area were deemed by the pathologist to be more closely associated with changes related to the presence of a tumor. Information concerning treatment induced non-neoplastic dermal changes was determined from dosed skin that had no apparent masses or lesions.

2.10. Statistical evaluations

The data in this report are compiled from four independent replicates. Each independent study was subjected to statistical analysis before compilation which included: means and standard deviations, one-way analysis of variance, Bartlett's test of homogeneity of variance, Dunnett's T test of significance, and Cochran and Cox's modified t test of significance. Bartlett's test of homogeneity of variance was performed whenever group comparisons were made. When the data were

homogeneous, Dunnett's test was performed. When the data were non-homogeneous, Cochran and Cox's modified t test was performed. The number of total tumors was analyzed utilizing χ^2 test (since the numbers of observed tumors would generally follow a Poisson distribution). These comparisons were performed utilizing the data analysis toolpak within Microscoft Excel, version 5.0a (Redmond, WA). The number of microscopically confirmed tumor-bearing animals was analyzed utilizing Fisher's Exact Test (McKee et al., 1990). This statistic was performed utilizing GraphPAD InStat, version 1.15 (San Diego, CA). The Kolmogorov–Smirnov statistical test was used for histopathology data and compared the cumulative distribution of severity scores of a test group to the distribution of the control group.

The compiled data were subjected to statistical analysis using SigmaStat Ver. 2.0.3 (SPSS Science, Chicago, IL). Group survival and body weight data comparisons were made after normality testing of the data by the Kolmogorov–Smirnov test ($p < 0.05$). If the data were normally distributed and passed the Equal Variance test, they were subjected to one-way analysis of variance ($p < 0.05$) followed by multiple comparison analysis using Dunnett's test ($p < 0.05$). If the data did not pass normality testing, analysis was by the Kruskal–Wallis one-way analysis of variance on ranks. Groups 3, 4, and 5 were compared to Group 2 and Group 6 was compared to Group 1. The mean number of total tumors and the mean number of tumor-bearing animals was analyzed as described for the individual studies. Groups 3, 4 and 5 were compared to Group 2 and Group 6 was compared to Group 1. Dose–response was determined by comparing Group 4 to 3 and Group 5 to 4 and 3. Statistical tests were carried out at the $p < 0.05$ two-sided significance level.

There were few differences in statistical significances between the analysis of the individual studies and the combined data analysis. The differences did not affect the conclusions presented in this manuscript.

3. Results

3.1. Analysis of CSC from 1R4F cigarettes

When smoked under standard FTC conditions, the 1R4F cigarette is reported to produce 10.8 mg total particulate matter (TPM), 9.2 mg "tar," 0.8 mg nicotine, and 11.6 mg carbon monoxide (Sullivan, 1984). Smoke condensates from the 1R4F were analyzed for specific constituents to insure that the condensates from different collection periods maintained consistency. The small standard deviations of the data in Table 2 demonstrate the consistency of the smoke condensate collection method and its analysis. They also indicate the condensates used in these studies were similar enough to allow the results from the four independent studies to be compiled into a single data set.

3.2. Analysis of dosing solutions

Analyses of the dosing solutions to confirm dosing and insure the doses were as intended are presented in Table 3. All dosing solutions were within −1.5 to 6.1% of their intended concentrations.

3.3. Survival

Survival among the various groups within these studies is shown in Fig. 1. Survival was generally good with 96% of all study animals alive at the end of the studies. Mice that did not survive to termination of the studies included those that died shortly after dosing (15%), those sacrificed for humane reasons (55%), those found dead without overt causation (27%), and those dying from other causes (3%). Mice that died shortly after dosing did so generally early (weeks 1–6) in the study. Later in the studies (weeks 20–30), mice were generally sacrificed for humane reasons. These sacrifices were performed primarily as a response to the development of larger masses

Table 2
Analysis of selected constituents of 1R4F cigarette smoke condensates used in dermal tumor promotion studies[a]

pH	Tar (mg/cig)	Nicotine (mg/cig)	Benzo(a)pyrene (ng/mg "tar")	Hydroquinone (μg/mg "tar")	Catechol (μg/mg "tar")	Phenol (μg/mg "tar")	*p*-,*m*-Cresol (μg/mg "tar")
5.7 ± 0.4	6.00 ± 0.55	0.48 ± 0.04	0.47 ± 0.12	3.0 ± 0.7	2.9 ± 0.9	0.4 ± 0.1	0.3 ± 0.1

[a] Data represent the mean and SD from four independent studies.

Table 3
Analysis of 1R4F dosing solutions used in 1R4F dermal tumor promotion studies[a]

Dosing solution	Target concentration	Actual concentration	Percent difference
DMBA	375 μg/ml	387 ± 7 μg/ml	3.1 ± 1.9
1R4F CSC (low-dose)	10 mg/application	10.6 ± 0.2 mg/application	6.1 ± 1.4
1R4F CSC (mid-dose)	20 mg/application	20.7 ± 0.4 mg/application	3.5 ± 1.9
1R4F CSC (high-dose)	40 mg/application	39.4 ± 0.4 mg/application	−1.5 ± 0.9

[a] Data represent the mean and SEM from four independent studies.

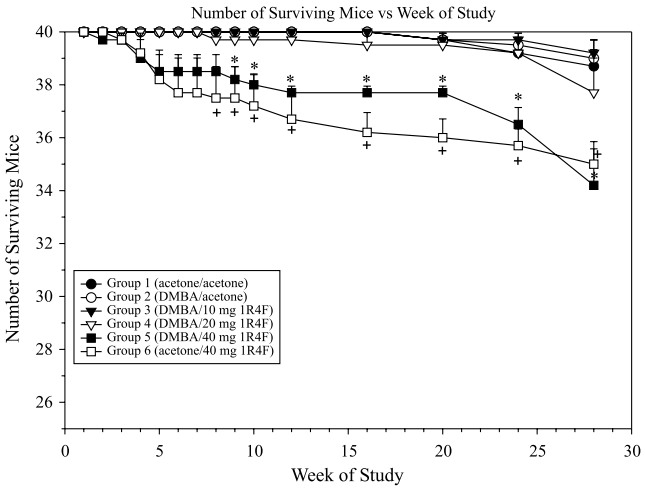

Fig. 1. Number of surviving mice over the course of the studies. Treatment regimen represented by (initiation treatment/promotion treatment). Data significantly different from the DMBA/acetone control (Group 2) are indicated by * and data significantly different from the acetone/acetone control (Group 1) are indicated by +.

or open sores that were affecting the general health and well-being of the animal.

The lowest survival rate was seen in the DMBA/1R4F 40 mg "tar"/application and in the acetone/1R4F 40 mg "tar"/application groups (approximately 86 and 88%, respectively). There were no significant differences seen between the mid- and low-dose 1R4F CSC treated groups (approximately 94 and 98%, respectively), although there was a trend toward the mid-dose having a slightly lower survival rate than the low-dose and controls. Between weeks 6 and 20, the rate of loss of mice in the high-dose 1R4F treated groups declined. This suggests an initial toxicological challenge to the mice in the high-dose groups followed by partial adaptation to the toxicity of the CSC. During this initial toxicological challenge, there was a trend toward DMBA treated mice demonstrating less sensitivity to CSC toxicity. This trend has been seen in other studies from this laboratory and indicates that DMBA treatment may reduce the sensitivity of the mice to CSC toxicity.

3.4. Clinical observations

Observations typical of dermal mouse studies and not attributable to treatment were noted throughout the studies. These included occasional observations of unkempt hair and alopecia, abrasions/lesions noted outside the dosed area of the skin (i.e., limbs/tail, ears), and observations associated with the eyes (i.e., lens or corneal opacity). These observations occurred in few mice randomly across all groups.

Abnormalities attributed to dermal CSC treatment were observed on the dosed skin. These included erythema, desquamation, peeling skin, and sores. These changes were considered CSC treatment-related because they were not observed in the acetone/acetone and DMBA/acetone groups. They occurred, in most part, in a dose-dependent manner with mice treated with 40 mg "tar"/application 1R4F CSC (either acetone or DMBA initiated) having the greatest incidence and duration.

Abrasions or lacerations of the dorsal skin resulting from hair clipping were noted sporadically throughout the studies in all groups. Most of these healed within 1–3 weeks. Since it has been reported that repeated abrasion of initiated skin can be sufficient stimulus for tumor promotion (Argyris and Slaga, 1981), the location of abrasions/lacerations was compared to skin mass location at necropsy. No correlation between abrasion/lacerations and mass location was observed.

3.5. Body weights of mice over the course of the study

Group mean body weights increased over the course of the studies as shown in Fig. 2. There were neither significant differences between the CSC treated mice and their respective controls nor were there dose-related differences between treated mice. Within any of the four independent studies, there were occasions when statistically significant differences in weekly group mean body weights between the treated and control groups were detected. These were not considered toxicologically significant because they were sporadic, occurred only occasionally and occurred in different groups at different times.

3.6. Dermal masses during the in-life phase of the study

During the in-life phase of the studies, masses were not categorized as to their potential diagnoses, such as papillomas or carcinomas, to avoid the subjectivity

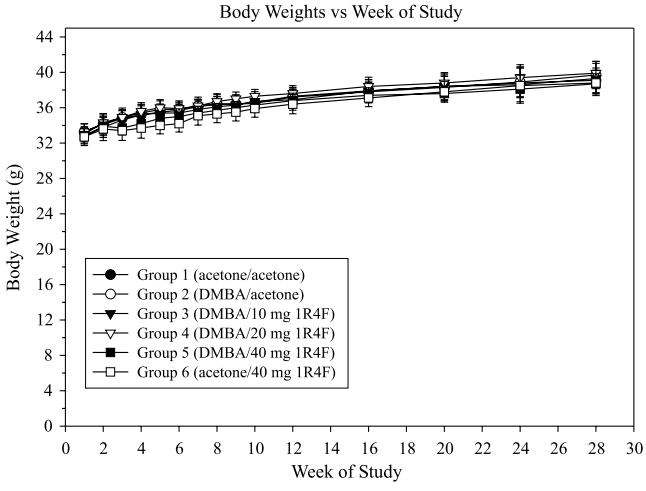

Fig. 2. Body weights of mice over the course of the studies. There were no statistically significant differences ($p < 0.05$) between any of the treatment groups.

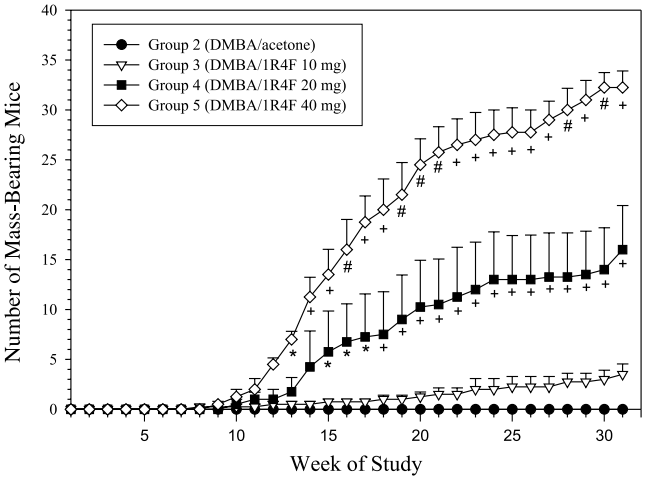

Fig. 3. Number of mass-bearing mice from the DMBA-initiated groups over the course of the studies. Data significantly different from the DMBA/acetone control (Group 2) are indicated by *. Data significantly different from the DMBA/acetone control (Group 2) and DMBA/low-dose CSC Group 3 are indicated by +. Data significantly different from the DMBA/acetone control (Group 2), the DMBA/low-dose CSC Group 3 and the DMBA/mid-dose CSC Group 4 are indicated by #.

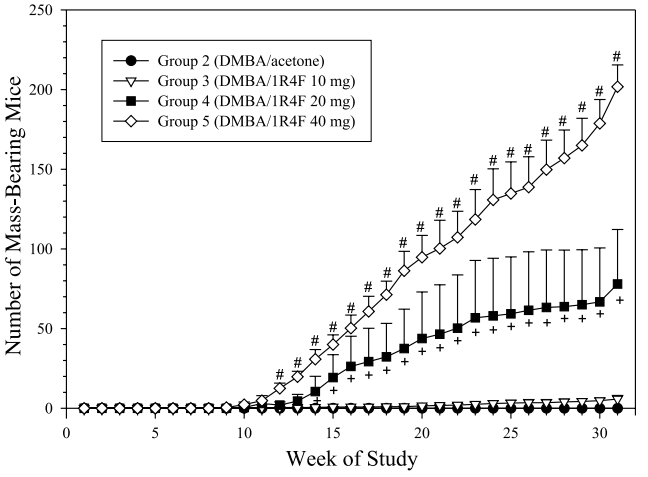

Fig. 4. Total number of masses from the DMBA-initiated groups over the course of the studies. Data significantly different from the DMBA/acetone control (Group 2) and DMBA/low-dose CSC Group 3 are indicated by +. Data significantly different from the DMBA/acetone control (Group 2), the DMBA/low-dose CSC Group 3, and the DMBA/mid-dose CSC Group 4 are indicated by #.

associated with mass classification without histological confirmation. No visible dermal masses were seen in the acetone/acetone and DMBA/acetone controls. The mean number of mass-bearing mice is provided in Fig. 3. At the low-dose, 1R4F CSC did not statistically significantly increase the mean number of mass-bearing mice when compared to the DMBA/acetone control, although there was a trend toward a small increase. At the mid-dose, there were statistically significant increases in the mean number of mass-bearing mice compared to the DMBA/acetone control starting at week 15 and continuing to study termination. Also, from week 18 to termination, the mid-dose 1R4F CSC treatment group showed a statistically significant increase compared to the low-dose CSC group. The mean number of mass-bearing mice was statistically significantly increased at the high-dose compared to the DMBA/acetone control from week 13 to termination. Also, from week 14 to termination, the high-dose group had significantly higher mean numbers of mass-bearing mice than the low-dose group and significantly higher mean number of mass-bearing mice at weeks 16, 19, 20, 21, 28, and 30 than the mid-dose group.

As seen in Fig. 4, at the low-dose, 1R4F CSC did not statistically significantly increase the mean number of total masses, although there was again a trend toward a small increase. At the mid-dose, there were statistically significant increases in the mean number of total masses compared to both the DMBA/acetone control and the low-dose CSC groups starting at week 14 and continuing to study termination. The mean number of total masses was statistically significantly increased at the high-dose compared to the DMBA/acetone control, the low-dose and mid-dose 1R4F from week 12 to termination.

These data demonstrate a definitive dose–response while the separation in response between the doses indicates they are appropriate for potential studies comparing various cigarette designs. Tightly spaced dose–response curves would decrease the sensitivity of the assay to detect changes between two cigarettes.

The acetone/high-dose 1R4F CSC treated group demonstrated a statistically significant increase in the mean number of mass-bearing mice (Fig. 5) at week 30

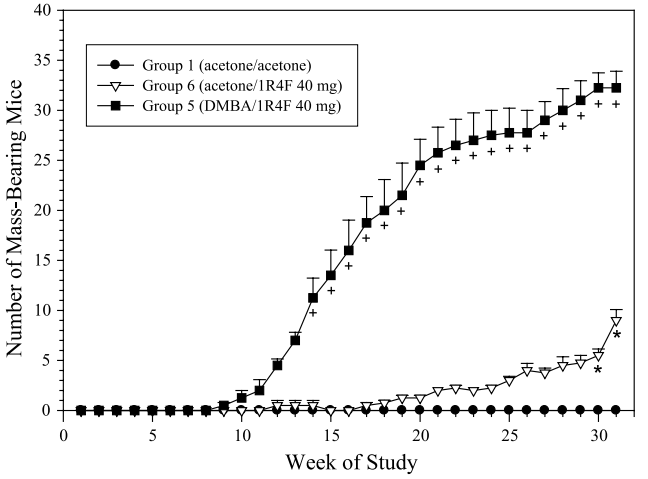

Fig. 5. Number of mass-bearing mice from the DMBA- and acetone-initiated high-dose CSC promoted groups over the course of the studies. Data significantly different from the acetone/acetone control (Group 1) are indicated by *. Data significantly different from the acetone/high-dose CSC (Group 6) are indicated by +.

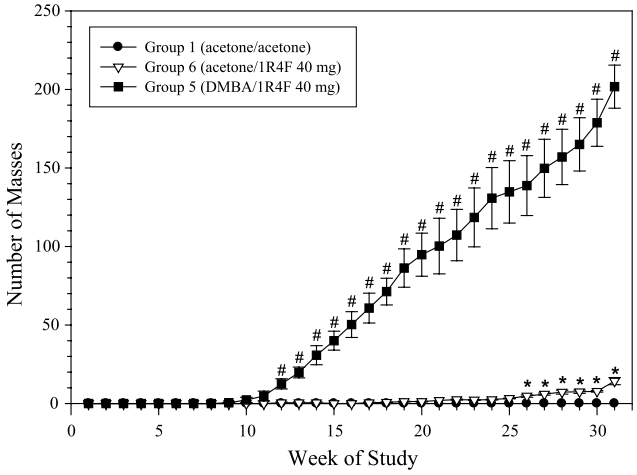

Fig. 6. Total number of masses from the DMBA- and acetone-initiated high-dose CSC promoted groups over the course of the studies. Data significantly different from the acetone/acetone control (Group 1) are indicated by *. Data significantly different from the acetone/high-dose CSC (Group 6) are indicated by #.

to 31 and a statistically significant increase in the mean number of total masses (Fig. 6) from week 26 to termination. These increases were statistically significantly smaller than those seen with DMBA/high-dose 1R4F CSC but do agree with previous studies indicating CSC is a weak initiator in the mouse dermal initiation/promotion assay.

As would be expected when using an outbred strain of mouse, tumor multiplicity (as measured by the number of masses per mass-bearing mouse) is variable. In the groups of 40 DMBA/high-dose CSC animals from these four studies, 4–10 responded by developing no masses while 2–5 developed 20 or more masses. The mean number of masses per mass-bearing mouse in the DMBA/high-dose 1R4F CSC group is approximately six.

3.7. Necropsy and histological analysis of selected organs

At necropsy, there were incidental findings commonly encountered in studies with mice. These included atelectasis of the lungs, thymic enlargement, and ovarian cysts. These occurred sporadically, across all groups and none appeared associated with treatment. Histological analysis of the brain, heart, liver, lungs, kidney, spleen, adrenals, ovaries, and gross lesions from the high-dose groups and controls indicated no definitive treatment-related trends. There was a statistically significant increase in extramedullary hematopoiesis of the spleen in the DMBA/high-dose 1R4F CSC groups compared to the DMBA/acetone groups in three of the four studies. However, this increase was seen only once when the acetone/1R4F CSC group was compared to the acetone/acetone control. Likewise, increased liver inflammation was seen in three of the four studies when the acetone/1R4F CSC treated group was compared to the acetone/acetone group but only once when the DMBA/1R4F CSC treated groups were compared to the DMBA/acetone groups. Therefore, no definitive conclusion can be drawn concerning whether or not these changes were related to 1R4F CSC treatment.

3.8. Histological diagnosis of non-neoplastic skin at treatment and non-treatment sites

Statistically significant histological changes that were dose-dependent occurred in DMBA-initiated mice promoted with 1R4F CSC at the mid- and high-dose. At the treatment site, these changes were characterized by acanthosis, generally accompanied by hyperkeratosis. At the inguinal site, hyperkeratosis was noted, but rarely accompanied with acanthosis. Whether or not the hyperkeratosis at the inguinal site was a generalized skin response or a response produced by spreading the 1R4F CSC to this site by grooming cannot be ascertained from the data.

When the acetone/1R4F CSC groups were compared to the acetone/acetone group the results were similar to those seen with the DMBA-initiated and 1R4F CSC promoted mice. These data indicate dermal treatment of mice with CSC produces acanthosis, generally accompanied with hyperkeratosis, at the treatment site.

3.9. Microscopically confirmed dermal tumors

All masses occurring at necropsy were examined histologically for diagnosis. Microscopically confirmed dermal tumor data are presented in Figs. 7 and 8. Across all four studies, the acetone/acetone control groups contained only one mouse with a tumor. This tumor (a trichoepithelioma) was considered a random event not associated with treatment. No mice with tumors were found in the DMBA/acetone control groups.

Although a few mice in the DMBA/low-dose 1R4F treatment group had tumors, there was no statistically significant difference between the low-dose and controls (Figs. 7A and B). In contrast, the DMBA/mid-dose 1R4F demonstrated a statistically significant increase in the number of mice with tumors (Fig. 7A) and the total number of tumors (Fig. 7B) when compared to the DMBA/acetone control and when compared to the DMBA/low-dose 1R4F group. The DMBA/high-dose 1R4F group showed a similar pattern in the number of tumor-bearing animals (Fig. 7A). The DMBA/high-dose 1R4F group demonstrated a statistically significant increase in the total number of tumors, when compared to the DMBA/acetone control and both the low-and mid-dose groups (Fig. 7B).

The predominant neoplastic changes observed upon microscopic examination of dermal masses or lesions in the dosed skin were papilloma and squamous cell car-

cinoma. Other neoplastic changes included keratoacanthoma, mast cell tumor and papule. For comparative purposes, the numbers of benign and malignant lesions were combined into total neoplasms. Combining neoplasms is conducted because the incidence of carcinoma in these studies only reflects the number of malignant lesions that arise during the established experimental period (30 weeks). Total number of neoplasms followed the patterns seen in the number of tumor-bearing mice.

The acetone/high-dose 1R4F CSC (40 mg "tar"/application) treated group provides data concerning the ability of 1R4F CSC to act as a complete carcinogen (initiator and promoter) in mouse skin within the context of the 29-week exposure period. The number of tumor-bearing mice (Fig. 8A) and the total number of tumors (Fig. 8B) were both significantly increased compared to the acetone/acetone control. However, these responses were much less than those seen in the comparable DMBA/high-dose 1R4F CSC (40 mg "tar"/application) treated group.

4. Discussion

To obtain quantitative, reproducible data from dermal initiation/promotion studies, protocol design

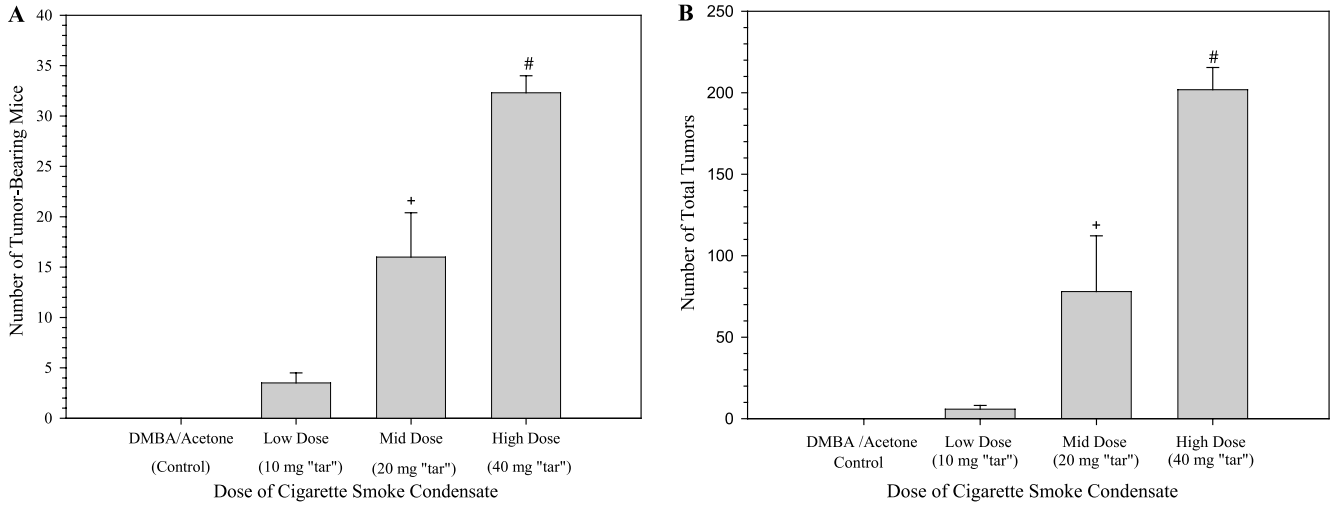

Fig. 7. DMBA-initiated groups over the course of the studies. (A) The number of microscopically confirmed tumor-bearing animals. Data significantly different from the DMBA/acetone control (Group 2) and DMBA/low-dose CSC Group 3 are indicated by +. (B) The total number of microscopically confirmed tumors. Data significantly different from the DMBA/acetone control (Group 2) and DMBA/low-dose CSC Group 3 are indicated by +. Data significantly different from the DMBA/acetone control (Group 2), the DMBA/low-dose CSC Group 3, and the DMBA/mid-dose CSC Group 4 are indicated by #.

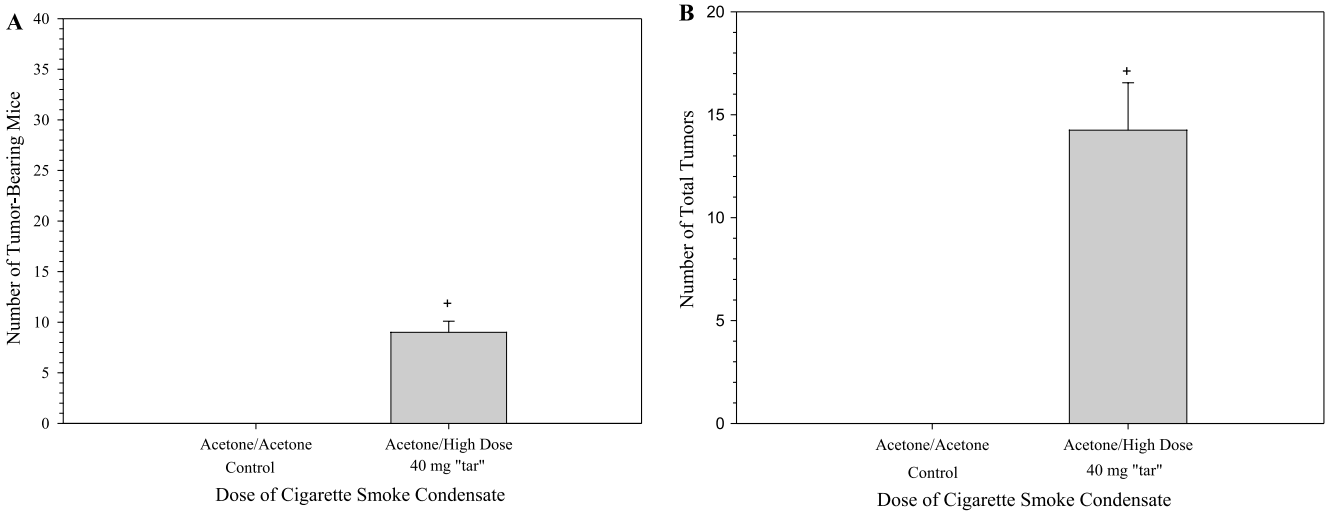

Fig. 8. The acetone-initiated high-dose CSC promoted groups over the course of the studies. (A) The number of microscopically confirmed tumor-bearing animals. Data significantly different from the acetone/acetone control (Group 1) are indicated by +. (B) The total number of microscopically confirmed tumors. Data significantly different from the acetone/acetone control (Group 1) are indicated by +.

standards need to be developed (Eastin, 1989; Schmidt and Hecker, 1989; Edler et al., 1991). A review of previous CSC studies using the mouse dermal initiation/promotion assay indicates that a number of factors are necessary to produce a reproducible, quantitative assay. First, CSC collection must be standardized to produce a material that is consistent and suitable for the comparison of CSC from various cigarettes. CSC is a chemically complex, metastable mixture that must be carefully collected and maintained to obtain consistency between batches. Second, the protocol should produce a reasonable and quantitative dose–response, which is consistent between studies and over time. Third, the assay should be rapid and efficient to provide useful data within a reasonable time. Fourth, the test species and strain should be readily available to the research community and be sensitive to the materials under evaluation. Fifth, the reference standard should have a consistent composition and design as well as produce reproducible, quantitative data in the assay and be readily available to other researchers. As shown below, the protocol presented in this report meets these criteria and provides a highly reproducible, quantitative study design to assess the promotional activity of CSC.

4.1. Standardized collection of CSC

The study of tumor promotion potential in dermal initiation/promotion assays requires large quantities of condensate. A critical concern in the design of these studies is how to collect the smoke condensate. A number of methods are available to collect the non-gaseous or particulate phase of cigarette smoke. These include cold trapping, filtration, electrostatic precipitation, and passing the whole smoke through various solvents, including water. As would be expected from a complex mixture, these methods produce preparations with different chemical and physical characteristics. Therefore, to obtain consistent, comparable data for an assay, a standard method must be selected and used. The chosen method should be relatively simple, use equipment readily available to most laboratories and minimize the number of chemical changes that occur between the smoke and condensate states. Cold trapping of smoke meets these requirements. This method was used by the National Cancer Institute in its Toward Less Hazardous Cigarettes program (NCI, 1980) and the data reported here indicate cold trapping yields a highly reproducible condensate. This reproducibility is an important contributing factor in the reproducibility of data from this protocol. Condensates produced by this method also have adequate storage stability to make the assay practical. The effects of storage on the tumorigenicity of condensate have been studied previously. Wynder et al. (1955) studied condensates stored from 1 to 6 months. Day (1967) studied condensates stored from 24 h to several weeks and Davies (1969) studied fresh (2- to 3-s-old) and 4-h-old condensate. These studies have shown no significant effect on the tumorigenicity of condensate as a result of storage and that the tumorigenic response is not the result of artifacts produced in the processing of condensate prior to storage.

4.2. Quantitative dose–response

An important criterion for a comparative assay is an adequate dose–response. The high-dose should provide an unequivocal positive finding with the reference cigarette but not yield unacceptable toxicity or low survival. As can be seen from the 1R4F data, the high-dose used in this standard protocol meets these criteria. In the reported studies, survival and toxicity (as indicated by body weight) were not adversely impacted. Another consideration is that the high-dose should not produce unacceptable high numbers of papillomas because of their effect on the health, well-being, and survival rates of the mice. The mid-dose in a comparative study should produce tumor numbers that are statistically above the control and low-dose but below the high-dose. Also, there should be enough difference between the response at the mid-dose and high-dose to detect condensates that may produce an increase or decrease in response at the mid-dose. The low-dose should provide a no-effect level or minimally, a very low response. Again, this increases the sensitivity of the assay towards condensates that may produce an increase in response. The data reported here confirm that the protocol utilized meets these criteria. These data also indicate that 1R4F CSC at doses of 40 mg "tar"/application may be a weak dermal initiator but is primarily a promoter of dermal neoplasm formation in the SENCAR mouse.

Useful data derived from this specific protocol include the total numbers of histologically confirmed tumors per treatment group and the total number of mice with tumors at the end of the 29-week promotion period. These end-points emphasize the total treatment group population instead of individual mice. The duration of the study allows sufficient time for papilloma development but a longer observation period would be required if maximum malignant neoplasm formation was the goal. The specific carcinoma incidence in these studies is not information that is interpretable in a meaningful manner since it can only reflect malignant lesions that arose during the 30-week experimental period. Carcinoma incidence is in a clearly ascendant slope (latency 20–60 weeks) in a study of this duration. More pre-malignant lesions are being recruited as new carcinomas at the 30-week termination (Reiners et al., 1984; Scribner et al., 1983). Since the number of carcinomas is less than those formed in longer studies and does not plateau, it is not possible to predict (at 30 weeks) whether the remaining papillomas will become malignant

(Aldaz and Conti, 1989). As a result, the quantitative biological significance of malignant changes based on this protocol is unknown. No biological significance, other than their contribution to the total tumor count, should be placed on the number of carcinomas observed.

4.3. Short-term results

Evaluation of the tumorigenicity of CSC has historically been accomplished with long-term studies that require between 1 and 2 years to conduct. Initiation/promotion assays typically are conducted for 52 weeks. The responsiveness of SENCAR mice in this tumor promotion assay provides an adequate response within 30 weeks. Data presented here indicate this response is consistent over time and shows little intra-laboratory variability. Both of these criteria are important in the development and utilization of a standardized protocol.

4.4. Test species and strain appropriate

To produce a consistent, comparative assay, the selection of animal species and strain must be considered. An ad hoc panel on chemical carcinogenesis assays established by the National Toxicology Program (NTP) (NTP, 1996) noted the mouse skin initiation/promotion model is more responsive (i.e., rapidly develops tumors) than other rodent models. Not all mouse strains are equally sensitive to dermal tumor promotion. $B6C3F_1$, Swiss (CD-1) and SENCAR mice were found sensitive to dermal promoters by developing tumors after topical application of either 7,12-dimethylbenz[a]anthracene or *N*-methyl-*N*′-nitro-*N*-nitrosoquanidine as initiators and 12-*O*-tetradecanoylphorbol-13-acetate or benzoyl peroxide as promoters. SENCAR mice were most sensitive based upon the lower doses that were generally required to produce effects equivalent to those in the other two strains. Tumors tended to develop earlier and with greater multiplicity in SENCAR mice than in the other two strains. The report concludes that SENCAR mice are the most acceptable strain for use in promotion studies (NTP, 1996), in agreement with an earlier Environmental Protection Agency (EPA) report (EPA, 1986). Nesnow et al. (1986) reported the inter-experimental variability of papilloma formation from a series of 25 experiments with SENCAR mice after 30 weeks promotion with tetradecanoylphorbol. Low statistical variation was observed in papilloma multiplicity, papilloma incidence, or papilloma latency. During the study, SENCAR mice of both genders had equivalent values for three variables: papillomas/mouse, mice bearing papillomas, and time when 10% of the mice bore papillomas. The lack of gender differences indicates there is no need to use both genders of SENCAR mice. The SENCAR mouse model provides adequate dose–response relationships for many tumor initiators and for many tumor promoters compared with other mouse strains (Slaga, 1986) and the higher sensitivity of SENCAR mice to the initiation/promotion regimen appears related to tumor promotion (Yuspa et al., 1996). Further support for the use of the SENCAR strain is based upon the large historical database of initiation/promotion studies using this strain (Slaga, 1986). These factors make SENCAR mice an ideal choice for this protocol. The results reported here confirm the SENCAR mouse is adequately responsive to DMBA initiation and CSC promotion for use in this protocol.

4.5. Reference standard

Standardized protocols should occasionally be checked against historical data. For CSC initiation/promotion protocols a reference cigarette is needed. A series of cigarettes has been developed jointly by the US National Cancer Institute, the Agriculture Research Service of the US Department of Agriculture, and the University of Kentucky Tobacco and Health Research Institute to serve as reference cigarettes for experimental purposes (Sullivan, 1984). These cigarettes incorporate the principal cigarette design advances that have been introduced into the marketplace since the 1950s and provide a basis for comparing data collected in different laboratories and at different points in time.

The 1R4F cigarette incorporates filtration, air dilution, and a tobacco blend that includes reconstituted tobacco sheet to achieve smoke yields typical of a Low Tar cigarette, on average, 9.2 mg "tar" and 0.8 mg nicotine when smoked under standard FTC conditions. Based on the tobacco blend, "tar," nicotine, and carbon monoxide yields (in relation to the US sales weighted average), the 1R4F was designed to be representative (on average) of the US cigarette market, in general, and the Full Flavor Low Tar (FFLT) segment, in particular.

In 1995, Steele et al. (1995) compared the mutagenicity of the total particulate matter of mainstream cigarette smoke from a representative sample of the US cigarette market with the 1R4F reference cigarette. The 1R4F, as assessed by the Salmonella assay, was representative of the US cigarette market with respect to mutagenicity. More recently the mainstream smoke yields of selected constituents from the 1R4F reference cigarette have been demonstrated to be representative of the average yields associated with the FFLT "tar" segment of the US cigarette market (Chepiga et al., 2000). Other studies have provided data concerning the chemical composition of 1R4F smoke and its TPM (Risner and Cash, 1990; Risner, 1988; RJRT, 1988a,b; White et al., 1990; Byrd et al., 1990; Nanni et al., 1990; Pryor et al., 1990; Rando et al., 1992; Borgerding et al., 1997; Daisey et al., 1998). Toxicological studies using the 1R4F cigarette have included genotoxicity (Doolittle

et al., 1990a,b), cytotoxicity (Lee et al., 1990; Bombick et al., 1998a,b; McKarns et al., 2000), DNA adduct formation (Lee et al., 1992a,b, 1993; Brown et al., 1997, 1998), effects on intercellular communication (McKarns et al., 2000), inhalation toxicology (RJRT, 1988b; Coggins et al., 1992), and inhalation oncogenicity (Witschi et al., 1997; Witschi, 1998). Overall, these results support the use of the 1R4F as an acceptable reference cigarette for comparative studies of cigarettes available in the US marketplace.

5. Conclusions

The data presented here indicate the 1R4F reference cigarette produces a consistent response in properly designed mouse dermal initiation/promotion studies. The protocol used for these studies produced highly reproducible data and demonstrated high sensitivity to CSC. There was a definitive dose–response. The doses used in these studies and the responses observed should enable the detection of biologically significant differences between CSC from different cigarettes, if present. Overall, these results support the use of the dermal tumor promotion protocol described in this report as an effective model that may be used to evaluate changes in cigarette design, new materials used in the manufacture of cigarettes, changes in tobacco processing, or the development of new technologies to increase or reduce the biological activity that results from burning tobacco.

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