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Recent Advances in the Toxicological Evaluation of Tobacco Products

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Contents

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PREFACE

This publication, **Recent Advances in Tobacco Science** Volume 28, continues the policy of publication of each year's Symposium that was initiated in 1975 at the 29th Tobacco Chemists' Research Conference. This series of conferences is now known as the Tobacco Science Research Conference, and information on ordering additional copies of this volume as well as previous volumes can be found on this last page of this volume.

This volume contains the five presentations that comprise this year's Symposium, "Toxicological Evaluation of Tobacco Products: Current Approaches, Future Developments," which was held on September 30, 2002, as part of the 56th Tobacco Science Research Conference in Lexington, Kentucky. The Program Editorial Committee chose the theme of the Symposium; and invited Dr. Scott Appleton, Director of Scientific and Regulatory Affairs, at Brown & Williamson Tobacco Corporation, to serve as Chairperson of the Symposium. He selected the general topics to be covered and invited experts in the areas to participate in the Symposium. Each author was given complete freedom in the development of the subject matter. We hope you find these presentations to be interesting and informative and that this volume will serve as a reference on the toxicological evaluation of tobacco products.

Cheryl Johnson, Charles S. Johnson, and I, this year's Program Editorial Committee, thank the authors and the Symposium Chairperson for their dedication and cooperation in preparing this publication.

John H. Lauterbach, Chairperson

Program Editorial Committee

56th Tobacco Science Research Conference

INTRODUCTION TO THE SYMPOSIUM

Scott Appleton

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I would like to welcome the speakers and audience to the 56th Tobacco Science Research Conference and to today's Symposium titled "Toxicological Evaluation of Tobacco products".

Adverse health effects resulting from the use of cigarettes and other tobacco products have been a concern of the public health community for over 40 years. It is therefore important to assess the potential health impacts of products employing novel materials or designs to assure such new design features do not increase the adverse health effects of using such products above and beyond those currently recognized for traditional tobacco products.

The process of how to conduct these assessments is far from an exact science or routine process. There are several reasons for this. One reason is that there is a great deal of scientific uncertainty about what characteristics of tobacco products play significant roles in tobacco related disease. Secondly, there are no known toxicological tests that are regarded as being predictive of risk outcome in people who use tobacco products. Unlike the situation with many other consumer products, there are no unified guidelines from the scientific or regulatory communities about what design characteristics of tobacco products need to be tested or how such testing should be conducted. Because of this, the process of evaluating the potential health impacts of new product design parameters entails a great deal of scientific judgment and

is typically applied on a case-by-case basis. Given the uncertainties of this endeavor, benchmarking methods employed by investigators in the scientific community would appear to be an appropriate strategy for identifying approaches that have proven useful in both the past and present.

Our Symposium will begin with Dr. John Lauterbach of the Brown & Williamson Tobacco Corporation. His paper, Smoke Chemistry: A Useful Predictor of Smoke Toxicology?" will review some of the historical relationships that have been developed between tobacco and smoke chemistry and various biological endpoints that have been associated with tobacco-related diseases. Smoke chemistry has the potential to identify the critical links between the product, its design, its performance characteristics, and smoking-related health risks. Through the 1950s, 60's, and 70's advances in the analytical chemistry of cigarette smoke identified constituents such as benzo(a)pyrene, HCN, phenols, CO, nitrosamines, and others as potentially playing significant roles in tobacco related disease. These approaches were pioneered by investigators like Dr. Dietrich Hoffman of the American Health Foundation. In addition, Dr. Lauterbach will identify relationships between various cigarette design parameters, their influence on smoke chemistry, and how they affect biological activity. Finally, he will discuss strategies for handling detailed smoke chemistry data.

It is widely accepted that a critical molecular event in cancer initiation is some form of genetic damage. In the late 1970's *in vitro* tests for mutagenic activity began coming into use as tests of a biological endpoint believed to have a mechanistic link to cancer initiation. The reverse mutation assay in Salmonella developed by Bruce Ames is the most widely accepted test of this type. Investigators such as Kier and Mizusaki began applying this test to the assessment of cigarette smoke in the late 1970's. Dr. Eian Massey from British American Tobacco Company will discuss *in vitro* tests for two different types of genetic damage, namely, gene mutations in

bacterial cells as measured by the Ames test and structural and numerical chromosomal changes as measured by the micronucleus test. After reviewing the biological basis of these tests, Dr. Massey will discuss the present state of knowledge about various cigarette design variables, specific constituents in tobacco smoke, and how they can influence activity of cigarette smoke in these tests. Alternative methods of cigarette smoke collection and presentation to various systems to better simulate whole smoke exposure will also be presented.

Dr. David Bombick from R. J. Reynolds Tobacco Company will discuss the use of *in vitro* test systems as measures of cell cytotoxicity. Cytotoxicity is believed to play an important role in both nonneoplastic and neoplastic smoking related diseases. Therefore, *in vitro* tests for cell toxicity are an important compliment to *in vitro* measures of genetic toxicity. Beginning in the 1960's and continuing through the 1970s, measurements of ciliatoxicity of tobacco products were made by investigators such as Battista and Dalhamn. These early studies began the use of *in vitro* cytotoxicity tests as measures of potential harm from cigarette smoke. Dr. Bombick will discuss the application of the Neutral Red test for evaluation of modifications of conventional cigarettes as well as potentially reduced risk cigarettes. In addition, tests based upon a variety of specific physiological processes within the cell will be reviewed for their possible use in assessment of tobacco products.

Dr. Daniel Heck of the Lorillard Tobacco Company will present the major types of animal models used for assessment of tobacco products. The use of living animal models has long been employed as a means of replicating, as closely as possible, the way humans are exposed to tobacco and tobacco smoke, as well as the complex biological processes that ultimately result in smoking related diseases. Through the 1960's, 1970's, and 1980's various *in vivo* toxicity tests such as dermal carcinogenicity and smoke inhalation tests in rodents were employed to

characterize the pathological changes produced by exposure to tobacco products. These techniques were employed by investigators such as Ernst Wynder, Fred Bock, The NCI's Tobacco Working Group, Al Wehner, Carol Henry, and the Leuchtenbergers. Dr. Heck will discuss some of the more important methodological aspects of these tests as well as their strengths and weakness for assessing the toxicological potential of tobacco products. Finally, Dr. Heck will discuss newly developed transgenic animal models and innovative smoke inhalation exposure protocols regarding their potential to enhance our ability to assess toxicological properties of tobacco products.

The recent publication of the Institute of Medicine report "Clearing the Smoke: Assessing the Science Base for Tobacco Harm Reduction" brought to focus the importance of assessment tools employing humans. A key emphasis of the report was the use of biological markers of exposure and harm resulting from use of tobacco products. Dr. Carr Smith of the R. J. Reynolds Tobacco Company will give a presentation on use of clinical evaluations to assess health effects of cigarettes developed for their potential to reduce smoking-related health risks. Dr. Smith will discuss details of both the design and conduct of various types of human studies. He will discuss various clinical markers for assessing both exposure and potential health effects resulting from cigarette use as well as the biological basis for markers of potential harm.

Looking at the approaches that have been presented today, it is clear that benchmarking approaches used by investigators within the scientific community has indeed generated practical and valuable guidance about appropriate methods for the toxicological evaluation tobacco products.

On behalf of the 56th Tobacco Science Research Conference, I would like to thank the speakers, Program Editorial Committee, and participants for their efforts in making this Symposium possible.

SMOKE CHEMISTRY: A USEFUL PREDICTOR OF SMOKE TOXICOLOGY?

John H. Lauterbach

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SUMMARY

Smoke chemistry, as a predictor of smoke toxicity, seems to have fallen into disfavor in recent times. First, industry critics claim that traditional smoking protocols seriously under estimate the doses of smoke constituents received by smokers, particularly for light and ultralight cigarettes. Second, the recent Institute of Medicine Report, "Clearing the Smoke," put emphasis on *in vitro* toxicology, human biomonitoring, and epidemiology as measures of harm reduction for Potentially Reduced Exposure Products (PREPS). Third, even if one obtains detailed smoke data under various conditions that may represent the extremes of human smoking behavior, one still has to decide what to do with the data, particularly if comparisons of potential biological activity are to be made among several products or a representative sample from a given market. However, not all is lost; there are a number of examples in the literature and company reports that show the potential relationships of product chemistry with biological endpoints. Furthermore, since smoke chemistry changes with the different smoking regimens such changes also may impact the *in vitro* biological assays, which are favored by some experts, as these rely upon essentially the same smoke collections used for chemistry studies. Finally, strategies for handling detailed smoke chemistry data will be discussed.

INTRODUCTION

Smoke chemistry, as a predictor of smoke toxicity, seems to have fallen into disfavor in recent times. First, industry critics claim that traditional smoking protocols seriously under estimate the doses of smoke constituents received by smokers, particularly for light and ultralight cigarettes (1, 2, 3). Second, the recent Institute of Medicine Report, "Clearing the

Smoke," put emphasis on *in vitro* toxicology, human biomonitoring, and epidemiology as measures of harm reduction for Potentially Reduced Exposure Products (PREPS) (4). Third, detailed chemical investigations of commercial smokeless tobacco products, cigarettes, or the smoke from those cigarettes can give hundreds of analytes, many of which appear at similar levels in what appear to be very dissimilar products, thus thwarting most attempts to link chemistry with biological endpoints. Still, numerous authors have put forward hypotheses linking smoke and tobacco chemistry to diseases that have been associated with tobacco use. Many others have reviewed the literature on possible relationships among tobacco and/or smoke chemistry and the toxicology of tobacco products. Examples of recent reviews include those by Hoffmann, Hoffmann, and El-Bayoumy (5), Rodgman, Smith and Perfetti (6), and Hecht (7).

Of all the measures of tobacco and/or smoke toxicity, chemistry is probably the easiest to do. It does not require human subjects or other mammalian species. It does not require special mammalian or bacterial cell lines and those with the special training to do *in vitro* or *in vivo* assays. With the exception of needing a top-notch smoking machine, which is required for all but smokeless products and human biomonitoring studies, one can do tobacco and smoke chemistry with fairly common instrumentation, instrumentation that we would likely have on hand for general tobacco and cigarette product research, and instrumentation that many chemists know how to use. Indeed, many tobacco scientists have made their careers on being able to identify the chemistry behind subtle differences in the hedonic properties between apparently very similar smokeless or smoking products. Therefore, where have we gone wrong, if indeed, we have gone wrong, in the use of chemistry to predict the toxicology of tobacco products?

In this paper, approaches will be given to meeting the challenges of relating tobacco and/or smoke chemistry with the toxicology of tobacco products. Examples will be taken from the peer-review literature, government reports, and company reports. In this latter regard, this

paper will include citations to the tobacco company document Internet sites that were established as part of the Master Settlement Agreement. These documents can also be accessed through links from other Internet sites such as www.tobaccoarchives.com and www.tobaccodocuments.org. These Internet sites also contain copies of public domain documents that may not be readily available from other sources.

If we go back in time to the early 1990s, we find that smoke chemistry was an important part of the program proposed by the U.S. Consumer Product Safety Commission for the assessment of the toxicology of cigarettes with reduced ignition propensity (8). At least one publication describes the comparison of the smoke chemistry and mutagenicity of two low ignition propensity cigarettes with commercial and reference cigarettes (9).

It was recognized that smoking topography could be different among different products and that smoke collection for both analytical and in vitro biological determinations should be done under several smoking regimens that the authors claimed would cover the range of expected smoking behaviors. Another key aspect of the proposed testing protocol was that it was designed to see if products, which incorporated technologies for reduced ignition propensity, gave a more toxic smoke than did conventional products. However, the manner in which the data from such comparison studies would be assessed was not specified. Perhaps those authors realized the conundrum an assessment body would be in if the product that had been modified for reduced ignition propensity showed, relative to the control product, more toxicity on some assays, but less toxicity on others. Obviously, if relative to the control product, the toxicity were higher on all assays (rejection of the test design) or lower on all assays (acceptance of the test design), the assessment would be easy. However, even among the *in vitro* and *in vivo* tests there can be differences. For example, all flue-cured cigarettes generally have higher biological activity than do all burley cigarettes in mouse skin painting assays for condensate tumorigenicity (10), while the converse is true for condensate mutagenicity as measured by the Ames assay (11). Furthermore, since the correlations between chemistries of

complex mixtures and the toxicology of those mixtures are often fuzzy, the toxicological significance of reductions of compounds or classes of compounds in the matrix is likely fraught with uncertainty.

It is not surprising then that much of the recent literature relating tobacco and smoke chemistry of conventional tobacco products with toxicological endpoints has been focused on showing that various design factors (i.e., tobacco additives, cigarette design features) do not increase toxicity. When considering conventional cigarette products, the data package will often contain routine tobacco and smoke chemistries, Hoffmann analytes, one or more in vitro assays for genetic and/or cytotoxicity, and a 13-week subchronic rodent inhalation study (12, 13, 14). Sometimes the inhalation studies are supplemented with a mouse skin painting study (15)

Outside of the major efforts associated with the assessment of non-conventional cigarette products such as Premier (16), Eclipse (17), and Accord (18), there is little in the recent literature to guide us in developing relationships among tobacco and smoke chemistries and toxicological endpoints for PREPS based largely on conventional technologies. Furthermore, if we look to the assessments that were used for Premier, Eclipse, and Accord for guidance, we find that there is little in the way of statistical correlations among various chemical parameters and toxicological endpoints.

The next part of this report will take examples from the literature to document the rightful place of chemistry in the assessment of the toxicological properties of tobacco products. It will begin with a review of the Tobacco Working Group studies of the 1970s (19). This will be followed by three examples from the literature where differences in tobacco and/or smoke chemistry have been liked to the epidemiology of tobacco-related disease. The final part of this report will cover some examples of the relationships between smoke chemistry and various *in vitro* and *in vivo* assays. In addition, examples for assessing and reporting detailed chemical data will be given along with some pitfalls in making correlations with biological endpoints.

DISCUSSION

The Tobacco Working Group

One of the great efforts in comparing chemistry data with toxicology data was the research program conducted by the Tobacco Working Group (TWG) (20, 21, 22, 23, 24, 25). The TWG was a joint program between the major cigarette companies and the Smoking and Health Program of the U.S. Department of Health, Education, and Welfare. More details about the TWG can be found in Chapter 4 of Reference 19. The TWG program began in 1968 and continued into the late 1970s. Numerous variants in blend, additives, and design were evaluated not only in terms of tobacco and smoke chemistry, but also in terms of tumorigenicity (mouse skin painting) and several measures of ciliatoxicity and cytotoxicity. In one sense, the TWG was a study of PREPS.

Table I, which was adapted from Reference 24, shows the cigarettes that were made for the program. In Experiment I, the key variables were the use of stem and various reconstituted tobaccos along with variables on cut width, cigarette paper, and nitrate addition. Variables in Experiment II included expanded tobaccos, agronomic practices, and the use of the artificial tobacco substitutes, NSM (ATS-A) and Cytrel (ATS-B). The focus of Experiment III was on paper porosity, tobacco additives, filters, and additional work with ATS-A and ATS-B. Experiment IV retested several of the concepts from the earlier experiments as well as different processing conditions and agronomic factors.

It must be pointed out that the TWG's efforts were not the first in this area. Numerous other researchers had studied the relationships among tobacco chemistry, smoke chemistry, and various indices of smoke toxicity. Some studies were summarized in the classic work by Wynder and Hoffmann, *Tobacco and Tobacco Smoke; Studies in Experimental Carcinogenesis* (26) and The Proceedings of the 3rd World Conference on Smoking & Health (27).

Table I
Experimental Cigarettes Tested in Skin Painting Experiments

Var. No.	Cig. Code	Experiment I Cigarette Description
1	1	University of Kentucky Reference (KY1R1)
2	2	SEB I
3	3	SEB I, High porosity citrate paper (Schweitzer #505) (48 cm/min)
4	4	SEB I, Low porosity phosphate paper (Ecusta Reference A of Schweitzer Regular Verge) (11 cm/min)
5	5	SEB I, Nitrates added as KNO_3, to 2X natural level of SEB I
6	6	SEB I, cut coarse (20 cuts/in)
7	7	SEB I, cut fine (60 cuts/in)
8	8	SEB I, with low porosity phosphate paper and coarse cut (combination of codes 4 & 6)
9	9	SEB I, with high porosity citrate paper, high nitrate content, and fine cut (combination of codes 3 & 5 &7)
10	10	SEB I, Lamina only (only leaves of the formula used)
11	11	SEB I, Flue-cured Lamina only (only flue-cured leaves of formula used)
12	12	SEB I, Burley lamina only (only Burley leaves used)
13	13	SEB I, Stems only (only flue-cured and Burley stems used, rolled and cut)
14	14	SEB I, Stems only, made into RTS (reconstituted tobacco sheet) by Schweitzer paper process
15	15	SEB I, Stems and fines only, made into RTS by AMF slurry process
16	16	RTS of whole SEB I by Schweitzer paper process, no additives, medium density
17	17	RTS of whole SEB I by Schweitzer paper process, 7.5% wood pulp added, low density
18	18	RTS of whole SEB I by Schweitzer paper process, 7.5% wood pulp added, medium density
19	19	RTS of whole SEB I by Schweitzer paper process, 7.5% wood pulp added, high density
20	20	RTS of whole SEB I by AMF slurry process, no additives, medium density
21	21	RTS of whole SEB I by AMF slurry process, additives*, low density
22	22	RTS of whole SEB I by AMF slurry process, additives*, medium density
23	23	RTS of whole SEB I by AMF slurry process, additives*, high density

*In addition to glycerine 2.81% and invert sugar 5.31%, additives were:

Methocel	7.35%
Refined unbleached sulfite pulp	4.59%
Ethylhydroxyethyl cellulose	1.84%

Table I. (Cont.)

Var. No.	Cig. Code	Experiment II Cigarette Description
1	40	University of Kentucky Reference (KY1R1)
2	41	SEB I
3	42	SEB II
4	43	SEB II
5	44	SEB II
6	45	SEB II
7	46	ATS-A, 100%
8	47	ATS-A, 50% & SEB II, 50%
9	48	RJ Reynolds puffed SEB II (Freon expansion process)
10	49	Philip Morris expanded SEB II (ammonia-CO_2 expansion process)
11	50	Freeze-dried SEB II
12	51	Straight Burley, normal nicotine, normal nitrogen fertilization NN)
13	52	Straight Burley, low nicotine, normal nitrogen fertilization (LN)
14	53	Straight Burley, low nicotine, high nitrogen fertilization (LH)
15	54	Straight flue-cured, normal nicotine, normal nitrogen fertilization (NN)
16	55	Straight flue-cured, low nicotine, normal nitrogen fertilization (LN)
17	56	Straight flue-cured, low nicotine, high nitrogen fertilization (LH)
18	57	Blend 3 parts flue-cured, 1 part Burley (NN)
19	58	Blend 3 parts flue-cured, 1 part Burley (LN)
20	59	Blend 3 parts flue-cured, 1 part Burley (LH)
21	60	Hand suckered (no suckering chemicals used)
22	61	Fatty-alcohol - normal
23	62	Fatty-alcohol – 100X
24	63	ATS-B straight (100%)
25	64	ATS-B, 50% & SEB II, 50%

Note: ATS-A = Artificial Tobacco Substitute A (NSM) and ATS-B = Artificial Tobacco Substitute (Cytrel)

Table I. (Cont.)

Var. No.	Cig. Code	Experiment III Cigarette Description
1	40	University of Kentucky Reference (KY1R1)
1A	41A	SEB I
3	41B	SEB I
4	75A	SEB III
5	75B	SEB III
6	72	SEB III
7	73	SEB III
8	74	SEB III
9	76	SEB III (Schweitzer paper 489-14, Low porosity) (5 cm/min)
10	77	SEB III (Vergé 55 paper-high porosity) (60 cm/min)
11	78	SEB III (Schweitzer perforated paper-very high porosity) (100 cm/min)
12	80	SEB III No sugar, with humectant
13	81	SEB III With sugar, no humectant
14	82	SEB III With 1% cocoa, no sugar, no humectant
15	83	SEB III No sugar, no humectant (no casing)
16	84	SEB III With L&M additive #1 (Magnesium Nitrate, 5.72%)
17	85	SEB III With L&M additive #2 (Zinc Oxide, 7.09%)
18	86	SEB III With L&M additive #3 (Magnesium Nitrate, 5.61%; Zinc Oxide, 6.96%)
19	87A	SEB III Burley Blend, cased with sugar, no humectant
20	87B	SEB III Burley Blend, cased with sugar. no humectant
21	88	SEB III Burley Blend, no casing (no sugar)
22	89	SEB III With dilution filter
23	90	SEB III With dilution filter and Schweitzer perforated paper (100 cm/min)
24	91	SEB III With cellulose acetate filter
25	92	SEB III With Permanganate filter
26	93	ATS-A, 30% & SEB III, 70%, plus flavor
27	95	ATS-A, 30% & SEB III, 70%, plus flavor
28	97	ATS-B, 100% (old material, old dyes)
29	99	ATS-B, 100% (new material, no dyes)
30	0	ATS-B, 100% (old material, no dyes)
31	01	ATS-B (old material, no dyes) 50% & SEB III, 50% (casing applied to tobacco portion only)

Table I. (Cont.)

Var. No.	Cig. Code	Experiment III Cigarette Description
32	79	SEB III
33	94	ATS-A, 30% & SEB III, 70% plus flavor with Vergé 60 cm/sec paper
34	96	ATS-A, 50% & SEB III, 50% (casing applied to tobacco portion only)
35	98	ATS-B, 100% (old material, new dyes)
36	00	ATS-B, 100% (new material, new dyes)

Note: Ecusta 556 paper used on cigarette codes 72 thru 75, 79 thru 89, 91 thru 93, 96 thru 99, and on 00, 0, and 01.
Note: ATS-A is artificial tobacco substitute A (NSM) and ATS-B is artificial tobacco substitute B (Cytrel).

Table I. (Cont.)

Var. No.	Cig. Code	Experiment IV Cigarette Description
1	30	University of Kentucky Reference (KY1R1)
1a	38	SEB III (Same as Code 75, Series III, L&M)
2	32	SEB IV
3	14	SEB IV
4	29	SEB IV
5	04	SEB IV
6	33	SEB IV, RTS, PJS paper process, no additives
7	09	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber)
8	06	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber), no return of water soluble substances
9	02	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber), nicotine reduced by proprietary process
10	28	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber), nicotine reduced and added back in form of nicotine citrate to level of Code 32, SEB IV
11	10	SEB IV, RTS, AMF slurry process, no additives except 2.8% glycerine, 5.31% invert sugar
12	17	SEB IV, RTS, AMF slurry process, 13% additives (see Note 1)
13	22	SEB IV, RTS, AMF slurry process, 13% additives plus 6% pH adjustment (see Note 2)
14	25	SEB IV, RTS, AMF slurry process, extracted with hexane-isopropanol azeotrope, 13% additives (see Note 1)
15	18	SEB IV, RTS, AMF slurry process, extracted with isopropanol-water azeotrope, 13% additives (see Note 1)
16	12	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber), waxy substances reduced
17	03	SEB IV, RTS, AMF slurry process 57%; plus 30% calcium carbonate plus Note 1
18	35	SEB IV, RTS, AMF slurry process 27%; plus 60% calcium carbonate plus Note 1
19	16	SEB IV, RTS, AMF slurry process 27%, extracted with isopropanol-water azeotrope, plus 60% calcium carbonate plus Note 1
20	27	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber) plus 25% inorganic fillers (calcium carbonate 18%, clay 7%)

Table I. (Cont.)

Var. No.	Cig. Code	Experiment IV Cigarette Description
21	19	SEB IV, RTS, PJS paper process, 10% additives (cellulose fiber) H_2O_2 treated
22	15	SEB IV expanded stems, 100%
23	08	SEB IV expanded stems 50%, SEB IV 50%
25	24	Ecusta material 30%, SEB IV 70%
27	13	SEB IV nicotine removed
28	11	SEB IV nicotine at 0.5 mg/cig.
29	31	SEB IV nicotine at 1.0 mg/cig.
30	23	SEB IV nicotine at 1.5 mg/cig.
31	26	Burley leaf with full return of stem
32	37	Burley leaf RTS, AMF slurry process, 15% additives (see Note 3)
33	21	Burley HLC RTS, AMF slurry process, 15% additives (see Note 3)
34	20	Bright leaf with full return of stems
35	36	Bright leaf RTS, AMF slurry process, 15% additives (see Note 3)
36	05	Bright HLC RTS, AMF slurry process, 15% additives (see Note 3)
37	L8	Pesticide free tobacco
38	M6	Pesticide treated tobacco (See Note 4)
39	67	SEB IV, treated with PMO (1.5% by weight)
40	68	SEB IV, with special 100 cm/min paper

Note 1. Additives: Refined unbleached sulfite pulp 6.05%, Galacto-Mannan Gums 5.85%, Cellulose Ether Gums 0.52%, Dialdehyde Crosslinker 0.58%, Total 13.00%

Note 2 Additives: Same as Note 1. plus Sodium Hydroxide 3.20%, Citric Acid 2.80%, Total 6.00%

Note 3. Additives: Refined Unbleached Sulfite Pulp 6.25%, Triethylene Glycol 2.25%, Galacto-Mannan Gums 4.48%, Cellulose Ether Gums 1.42% Dialdehyde Crosslinker 0.60%, Total 15.00%

Note 4. Additives: The soil fertility, pesticide residues, and pesticides used on Codes L8 and M6 are listed in a separate document.

RTS = Reconstituted Tobacco Sheet

PJS = Peter J. Schweitzer

AMF = AMF, Inc.

HLC = Homogenized Leaf Cured.

PMO = 3-phenyl-5-methyl-1,2,4-oxadiazole (an anti-irritant)

A short critical review of these studies was written by Slaven and can be found at the Lorillard document web site (28). For those who would like more detailed information than was published in References 20, 21, 22, 23, 24, three of the status reports can be found on the World Wide Web (29, 30, 31). Additional details on the analyses of the smoke condensates for the first two sets of experimental cigarettes can be found in a March 1975 report by Guerin and Nettesheim (32). Details of the smoke chemistry related to animal exposure systems can also be found in that report.

In the summary report of the mouse skin painting studies, factors that increased tumorigenicity relative to the Standard Experimental Blend (SEB) (in a standard nonfilter design) were identified along with those that decreased tumorigenicity or had little effect (24). Chemistry data was not given directly in that summary report, but was summarized in numerous tables in the four reports (20, 21, 22, 23). Tables II and III give some examples of the findings from the mouse skin painting studies. Each table shows the cigarette factor tested, the survival probability based upon histopathologically verified tumor data for the 25-mg/day dose groups (PFH-25), the static burn rate (SBR) in units of mm/min, the sum of benzo[a]anthracene and benzo[a]pyrene in the condensate in units of ppm, and the cigarette code for the particular factor. Table II shows some of the examples where the factors reduced tumorigenicity relative to the respective SEB reference cigarette. Data are also included for the Kentucky 1R1 reference cigarette that was analyzed in each study group. These data are in Italics. Data from relevant comparison products are also shown in Italics. Table III shows similar data for factors that increased or did not alter tumorigenicity.

Referring now to Table II, the top few rows show some of the examples from TWG I (TWG I, TWG II, etc., designations for Experiment I, Experiment II, etc.). One of the main findings from TWG I was that lamina or mixtures of lamina and reconstituted tobacco sheet (RTS) such as the SEB I cigarette gave more tumorigenic condensate than did RTS alone. In

addition, stems were found to give condensate less tumorigenic than lamina. In general, such modifications gave products with higher static burn rates than the SEB I cigarette. The use of high porosity citrate paper gave a directional improvement in PFH-25. In TWG-II, the use of freeze-dried tobacco, one type of expanded tobacco as well as low nicotine tobaccos gave condensates that were less tumorigenic than the SEB II. The largest improvement was from the artificial tobacco substitute, ATS-A. The range of responses in TWG-III for PFH-25 was more limited than in the other experiments; however, the SEB III without sugar and humectant was the only test cigarette in that experiment giving a condensate significantly less tumorigenic at the 25-mg condensate dose level than was the normal SEB III. However, when tested at the 12.5-mg condensate dose level, this effect was not seen (22). The following quote was taken from Reference 22. "This suggests that at the lower dose, sugar and humectant in combination have little effect on condensate tumorigenicity but, at the higher dose level, sugar and humectant in combination may contribute to condensate tumorigenicity." In TWG-IV, tobacco reconstitution processes gave less tumorigenic condensates in some cases as shown for the two examples given in Table II.

Table III lists some of the cigarettes whose condensates had equal or greater tumorigenicity than did the corresponding SEB. In TWG-I, none of the combinations of cut width, paper porosity, and nitrate addition gave results statistically different from those of SEB I although directional differences were seen. In TWG-II, neither of the combinations of the SEB-II blend with ATS gave condensates whose tumorigenicity was different from that of the SEB II. The cigarettes made with the SEB II blend expanded by the Freon process gave condensate of comparable tumorigenicity with that of the SEB II, while the cigarettes whose tobacco was expanded by the ammonia-CO_2 process gave condensate less tumorigenic than that from the SEB II. TWG-III gave some interesting findings with respect to filters and dilution devices. While the cigarettes with the dilution filter, which was essentially a hollow tube with ventilation, gave a directional reduction in tumorigenicity, the cigarettes with the cellulose acetate filter and

the permanganate filter (cellulose/potassium permanganate on aluminum oxide/cellulose) gave statistically significant increases in tumorigenicity. One of the RTS samples in TWG-IV, which would have been expected to give reduced tumorigenicity based on its formulation, was found to give condensate whose tumorigenicity was not different from that of the SEB IV cigarettes.

TABLE II

SOME CIGARETTE FACTORS GIVING CONDENSATE LESS TUMORIGENIC THAN THE SEB

ADAPTED FROM REFERENCES 20, 21, 22, 23, 24

Factor	PFH-25	SBR	B[a]A+B[a]P	Code
SEB I, stems only, made into reconstituted tobacco sheet (RTS) by paper process	0.896*	9.09	1.95	14
SEB I, stems and fines only, made into RTS by slurry process	0.692	6.16	2.02	15
SEB I, whole blend, made into RTS by paper process, medium density, no additives	0.812*	6.41	1.54	16
SEB I, whole blend, made into RTS by slurry process, medium density, no additives	0.522	4.55	1.90	20
SEB I, citrate paper, high porosity	0.663	4.55	1.89	3
Flue-cured and burley stems from SEB-I blend	0.817*	5.62	1.94	13
KY1R1	*0.454*	*3.84*	*2.06*	*1*
SEB I	*0.517*	*4.19*	*1.92*	*2*
SEB II, ammonia-CO_2 expansion process	0.583*	6.30	1.10	49
SEB II, freeze-dried	0.573*	7.72	1.51	50
Straight burley, low nicotine, normal nitrogen fertilization	0.738**	9.28	1.27	52
Straight flue-cured, low nicotine, normal nitrogen fertilization	0.706**	6.10	1.41	55
ATS-A (Artificial Tobacco Substitute A) 100%	0.924**	NR	5.70	46
ATS-A, 50% & SEB II, 50%	*0.411*	*5.20*	*2.51*	*47*
KY1R1	*0.343*	*3.89*	*1.79*	*40*
SEB II	*0.442*	*4.97*	*1.64*	*42 - 45*
ATS-A, 70% & SEB III, 30%	0.557	4.17	3.63	93
SEB III with dilution filter	0.497	4.88	3.01	89
SEB III without sugar and humectant casing	0.594*	4.81	2.21	83
SEB-III with 5.72% magnesium nitrate	0.551	4.18	1.21	84
KY1R1	*0.434*	*3.76*	*2.34*	*40*
SEB III	*0.449*	*4.15*	*2.26*	*72-75*
SEB IV, whole blend, made into RTS by paper process, 10% fiber, no solubles add-back	0.814**	4.62	3.02	6
Bright leaf, made into RTS by slurry process, 15% additives (fiber, TEG, binders)	0.701**	6.05	1.68	36
Burley leaf, made into RTS by slurry process, 15% additives (fiber, TEG, binders)	*0.593*	*6.51*	*1.02*	*37*
KY1R1	*0.440*	*3.50*	*2.19*	*30*
SEB IV	*0.488*	*4.30*	*1.86*	*4, 14, 29, 32*

*p<0.05 **p<0.01 NR = Not reported

TABLE III

SOME CIGARETTE FACTORS GIVING CONDENSATE SIMILAR OR MORE TUMORIGENIC THAN THE SEB

ADAPTED FROM REFERENCE 24

Factor	PFH-25	SBR	B[a]A+B[a]P	Code
SEB I, phosphate paper, low porosity	0.565	3.55	2.29	4
SEB I, citrate paper, high porosity	*0.663*	*4.55*	1.89	*3*
SEB I, cut coarse (20 cuts/in)	0.449	3.93	2.31	6
SEB I, cut fine (60 cuts/in)	0.544	4.81	2.17	7
SEB I, with low porosity phosphate paper and coarse cut filler	0.532	3.27	2.12	8
SEB I, with high porosity citrate paper, fine cut filler, and 2x blend nitrate	0.654	5.84	1.75	9
KY1R1	*0.454*	*3.84*	*2.06*	*1*
SEB I	*0.517*	*4.19*	*1.92*	*2*
SEB II, Freon expansion process	0.542	6.32	1.64	48
Straight burley, normal nicotine, normal nitrogen fertilization	0.434	7.19	1.34	51
Straight flue-cured, normal nicotine, normal nitrogen fertilization	0.399	4.96	1.35	54
ATS-B, 50% & SEB II, 50%	0.455	5.62	1.86	64
ATS-A, 50% & SEB II, 50%	*0.411*	*5.20*	*2.51*	*47*
KY1R1	*0.343*	*3.89*	*1.79*	*40*
SEB II	*0.442*	*4.97*	*1.64*	*42 - 45*
SEB III with cellulose acetate filter	0.294*	4.65	3.50	91
SEB III with permanganate filter	0.298*	4.68	2.45	92
SEB III with dilution filter	*0.497*	*4.88*	*3.01*	*89*
SEB III with low porosity paper (5 cm/sec)	0.331	3.06	2.36	76
SEB III with high porosity paper (60 cm/sec)	0.407	4.49	2.43	77
SEB III with very high porosity paper (100 cm/sec)	0.413	4.62	2.21	78
KY1R1	*0.434*	*3.76*	*2.34*	*40*
SEB III	*0.449*	*4.15*	*2.26*	*72-74, 75A*
SEB IV, made into RTS by paper process, 10% fiber, 18% $CaCO_3$, 7% clay	0.394	6.06	2.71	27
KY1R1	*0.440*	*3.50*	*2.19*	*30*
SEB IV	*0.488*	*4.30*	*1.86*	*4, 14, 29, 32*

*p<0.05 **p<0.01

Considerable effort was made during the TWG studies to use statistical techniques to correlate the tobacco chemistry data, cigarette property data, and smoke chemistry data with the mouse skin painting data. These correlations are detailed in each of the four main TWG reports (20, 21, 22, 23). One of the more interesting correlations was a positive correlation between the nicotine content of the condensate and biological activity. Another one was that static burn rate was negatively correlated with tumorigenicity. It must be pointed out that the tumorigenicity assays were done on a dry condensate basis. Thus, the same amount of dry condensate was applied whether or nor the cigarette yield of dry condensate was 25 mg/cigarette or 10 mg/cigarette.

As pointed out by Slaven, some of the correlations did not make sense from a chemistry point of view (28). For example, various gas-phase analytes were used in some of the correlations with tumorigenicity of condensate. It was found that acetaldehyde, formaldehyde, nitrogen oxides, carbon monoxide, and acrolein were negatively correlated with tumorigenicity. Since tobacco nitrate levels were negatively correlated with tumorigenicity, and since the delivery of nitrogen oxides is very dependent on blend nitrate (33), the negative correlation of nitrate with tumorigenicity would be expected. The relationships with the other gas-phase variables and tumorigenicity appear to be less clear.

Bayne of the Oak Ridge National Laboratory took a different approach for the statistical analysis of the TWG data (34). Only data on condensate analytes determined in all four studies were used and only cigarettes containing 70% tobacco were considered. In addition, the data Bayne used from the fourth study were preliminary and differ to some extent from those finally published. Two prediction models were developed; and these are shown in Table IV, which is an adaptation of Table 9 of Reference 34. To test the simpler (e.g., Prediction Model Derived from Significant Linear Terms) of the two models, the relevant data in Bayne's report were reentered into an Excel workbook. A plot of observed PFH-values versus predicted PFH-values for the

entire data set is shown in Figure 1. As can be seen from the data in Table IV, Bayne developed second-order terms that improved the predictive power of his models. His basis to use second-order terms came from a correlation analysis that showed that there was no single variable had a correlation coefficient greater than r = 0.4 with the histopathological probabilities.

When one reviews the coefficients in Table IV, one notes that except for the terms involving [nicotine]^2, the coefficients of all the other terms involving nicotine are negative. This means that as the concentration of nicotine in the condensate increased, the tumorigenicity of the condensate also increased as measured by the decrease in the probability of tumor-free animals. However, nicotine is not mutagenic in the Ames assay and is it not tumorigenic (35, 36), but those factors had not been clearly established at the time of the TWG. Therefore, what was the mechanism underlying this very strong correlation between nicotine concentration in the condensate and tumorigenicity? One hypothesis was that other components in the condensate that may have arisen from the pyrolysis of nicotine were the culprits.

There was significant circumstantial evidence for that hypothesis. In 1961, Jarboe and Rosene of Brown & Williamson Tobacco reported that the pyrolysis of nicotine in an inert atmosphere at temperatures from 600ºC to 900ºC produced a variety of heterocyclic nitrogen compounds and aromatic hydrocarbons (37). In 1963, Kobashi, Hoshaku, and Watanabe reported on their pyrolysis studies of nicotine in air at temperatures ranging from 300ºC to 800ºC (38). Two fractions were collected in a cold trap. Fraction I (boiling points >200ºC) consisted chiefly of myosmine, pyridylmethylketone, isoquinoline, nornicotine, 2,2'-bipyridine, and nicotyrene. Fraction II consisted of 3-vinylpyridine, 3-cyanopyridine, other alkyl pyridines and pyrrole. In 1979, Schmeltz, Wenger, Hoffmann, and Tso reported on the comparison of the pyrolysis of ^{14}C-nicotine in a combustion tube versus that in a cigarette (39). Those authors reported that under pyrolytic conditions in a combustion tube, nicotine underwent extensive degradation to pyridines, quinolines, aryl nitriles, and aromatic hydrocarbons. However, when

the ^{14}C-nicotine was applied to commercial KS nonfilter cigarettes, over 40% of the nicotine remained intact (considering both mainstream and sidestream smokes).

Several other factors and hypotheses were presented in that era. During the Banbury Conference on Less Hazardous Cigarettes in October 1979, F. G. Bock's paper indicated that nicotine was a cocarcinogen (40). Hoffmann, Chen, and Hecht also presented a paper on their work on nitrosamines in tobacco and tobacco smoke (41). In addition, Mizusaki and coworkers had reported in 1977 that the levels of total nitrogen, protein nitrogen, and soluble nitrogenous material in leaf were positively and significantly related to an increase in the mutagenic activity of smoke condensate (42). However, those authors also reported that nicotine and nitrate were not important factors for the mutagenicity of the condensate. Furthermore, there were reports in the literature on the mutagenicity of carbolines in cigarette smoke (43, 44).

These factors and possibly others apparently led the German Verband der Cigarettenindustrie to commission research on the fate of nicotine in the burning cigarette, and that research became known as the N-PAH project (45, 46, 47, 48). One part of this project was research by Neurath to determine the amounts of a-, ß-, and ?-carbolines and aminocarbolines in cigarette mainstream and sidestream smoke (49).

The culmination of the N-PAH project appears to have been a report of research by a team of scientists that was lead by Robert Jenkins and Richard Izac of Philip Morris (50). This work used cigarettes made from tobaccos grown in a ^{14}C-CO_2 atmosphere. They reported between 0.13% and 0.32% of the total amount of ^{14}C-nicotine in a nonfiltered cigarette was converted to mainstream aza-arenes and between 0.14% and 0.3% of the original radiolabelled nicotine was converted to aza-arenes in the sidestream smoke. The authors did not comment on the biological activity of the aza-arene fractions that they isolated. Bleeker and coworkers have reviewed the toxicology of aza-arenes (51). Some of the compounds reported by Jenkins and Izac as pyrolysis products of the 14C-nicotine are known mutagens.

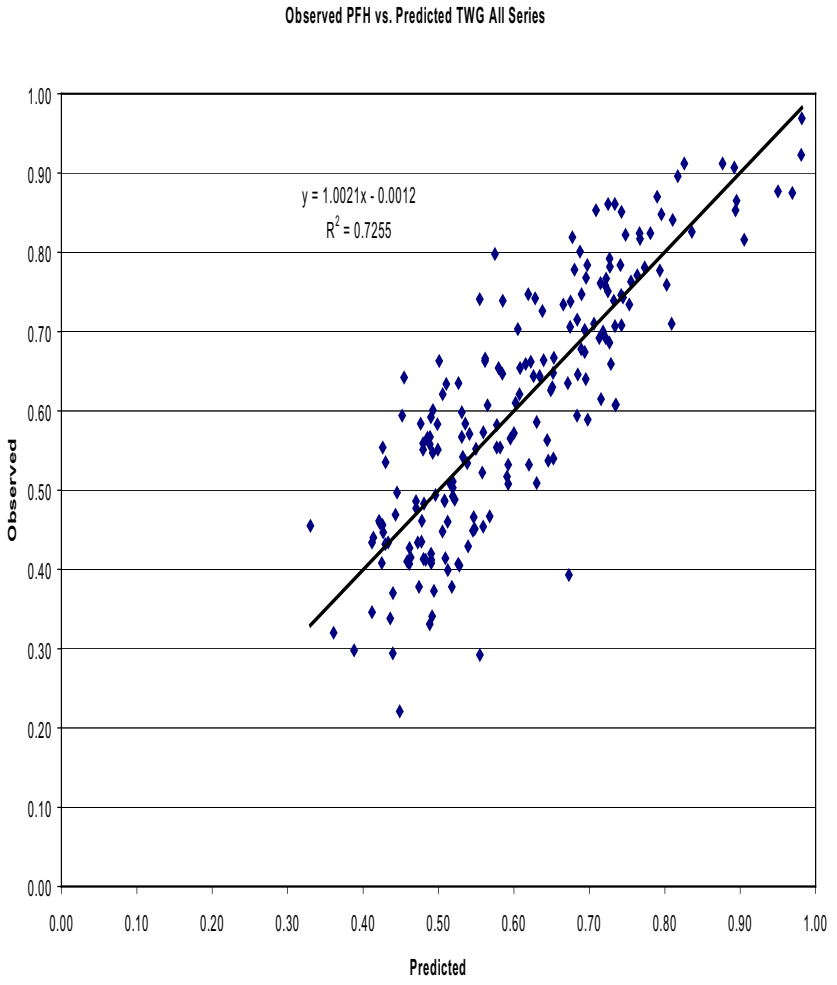
TABLE IV

COEFFICIENTS AND STANDARD DEVIATIONS OF COEFFICIENTS FOR PREDICTION MODELS OF TUMORIGENICITY

ADAPTED FROM REFERENCE 34

		Prediction Model Derived from the Full Second-Order Model		Prediction Model Derived from Significant Linear Terms	
Terms	Units	Coefficients	SD of Coefficients	Coefficients	SD of Coefficients
1 Intercept		3.065	0.347	2.637	0.292
2 Concentration	mg/day	-0.03716	0.00266	-0.03793	0.00274
3 $(\text{Concentration})^2$	$(\text{mg/day})^2$	0.0004613	0.0000392	0.0004688	0.0000408
4 Nicotine	mg/g	-0.006746	0.000830		
5 pH		-0.4221	0.1177	-0.4434	0.0980
6 Weak acids	meq/g	0.07786	0.03471		
7 Very weak acids	meq/g			0.1242	0.0555
8 B[a]P	μg/g	-0.7075	-0.244		
9 $(\text{Nicotine})^2$	$(\text{mg/g})^2$	0.00001512	0.00000532	0.00002450	0.00000588
10 $(\text{pH})^2$		0.02994	0.01063	0.03663	0.00875
11 $(\text{B[a]P})^2$	$(\mu\text{g/g})^2$	0.4318	0.1325		
12 Nicotine x Phenol	$(\text{mg/g})^2$	0.0008709	0.0002073		
13 Nicotine x pH	mg/g			-0.0007078	0.0001664
14 Nicotine x B[a]P	mg/g x μg/g			-0.001770	0.000377
15 B[a]P x Phenol	μg/g x mg/g	0.1378	0.0343		
16 o-Cresol x Phenol	$(\text{mg/g})^2$	-0.2797	0.0554		
17 o-Cresol x m&p-Cresol	$(\text{mg/g})^2$	0.5581	0.1153		
18 o-Cresol x B[a]P	mg/g x μg/g	-0.9991	0.2482		
19 o-Cresol x B[a]A	mg/g x μg/g	0.8453	0.1901		
20 m&p-Cresol x B[a]A	mg/g x μg/g	-0.3238	0.0678		

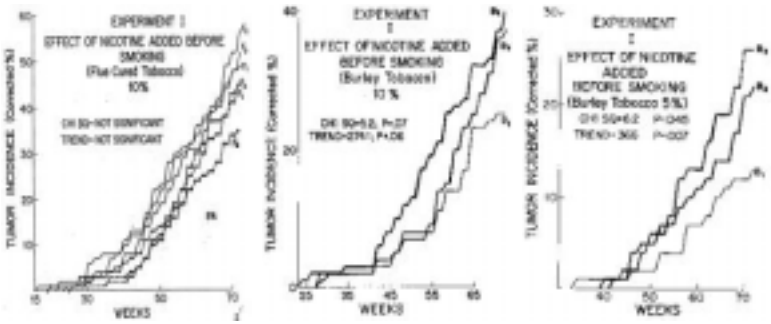
FIGURE 1


Observed PFH vs. Predicted TWG All Series
y = 1.0021x - 0.0012
R2 = 0.7255
Observed
Predicted
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00

Before leaving the subject of nicotine and tumorigenicity, another piece of evidence that was developed by Bock, Tso, and Fox was apparently presented at a scientific conference in 1981. The title of the presentation was, "The Effect of Nicotine on the Carcinogenic Activity of Cigarette Smoke." The slide copy can be found on the web site for the R. J. Reynolds Public Document Repository (52). The paper may have been presented at a conference in New York City in November 1981 (53).

In any case, cigarettes were made of either burley or bright tobacco containing equal mixtures of cured leaf and reconstituted sheet prepared from the same leaf. During the preparation of the reconstituted sheet, nicotine was added to the sheet providing low medium or high levels of nicotine of each tobacco type. According to the authors, the condensates from the bright tobacco exhibited twice as much carcinogenic activity as did the condensates from the burley cigarettes. However, the activity of the burley condensates depended on the nicotine content of the cigarettes while the activity of the bright condensates did not depend on the nicotine content of the cigarettes (52). Figures 2, 3, and 4 show the graphical representations of their data. The percentages shown in the Figures are the condensate concentrations.

Figure 2 Figure 3 Figure 4



These data show how the correlations of nicotine with tumorigenicity developed by the TWG did not take into account the underlying chemistry of the smoke in which the deliveries of actual tumorigenic agents were likely well correlated with nicotine deliveries.

A number of other reports and publications issued as result of the main TWG program. One such list of publications, reports, and presentations resulted from the TWG can be found in Reference 31. During the TWG studies, a number of advances in the assessment of tobacco smoke were applied to some of the TWG samples. One example was the determination of condensate mutagenicity by the newly introduced Ames assay (54). Table V shows a summary of the results along with the tumorigenicity (PFH-25), static burn rate (mm/min) and the sum of benzo[a]anthracene and benzo[a]pyrene concentrations in the condensate from Reference 21.

TABLE V

COMPARISON OF MUTAGENICITY AND TUMORIGENICITY OF SMOKE CONDENSATE

DATA ADAPTED FROM REFERENCES 21 AND 54

Cigarette	Mutagenicity CFU/mg condensate (TA1538+S9)	Mutagenicity rank	PFH-25	Tumorigenicity rank	Static burn rate (mm/min)	Sum B[a]A + B[a]P (ug/g)
KY1R1 (TWG II-40)	58.9	2	0.343	4	3.89	1.79
SEB II (TWG II-42)	99.9	4	0.442	3	4.84	1.66
Freeze-dried SEB II (TWG II-50)	47.1	1	0.573	2	7.72	1.51
Straight burley low nicotine normal fertilizer (TWG II-52)	67.5	3	0.738	1	9.28	1.27

The data in Table V show the conundrum often faced by smoke toxicologists. Different bioassays give different rankings of products. The mutagenicity data appear to show an effect of both blend and static burn rate. Based upon percentage of burley tobacco in the blend, the rankings, in decreasing order of mutagenicity, should have been II-52 > II-50 = II-42 > II-40 (11). Differences in static burn rate appear to have changed the expected rankings of mutagenicity.

Another aspect of the TWG was the study on selective filtration and the correlations of smoke chemistry with ciliatoxicity and cytotoxicity (25). Table VI shows the basic design and smoke parameters for the twelve cigarettes in that study. All cigarettes were 85 mm in length with a 20-mm filter section. In the case of F1, the filter section was a hollow tube while in the

case of F11, the filter section was a hollow tube with ventilation holes 10 mm from the mouth end. Table VII shows the other smoke analytes determined and the results of the ciliatoxicity, macrophage inhibition, and cytotoxicity assays. All values are expressed on a per-puff basis. The last column in Table VII shows the overall ranking given the different filter designs based on the bioassays (rank 1 least, toxic; rank 12, most toxic). Unlike the mouse skin painting experiments, the ciliatoxicity and macrophage inhibition bioassays were done with whole smoke. The cytotoxicity assay was done with the water-soluble portion of cold-trapped whole smoke. The results of these assays are expressed as ED_{50}/puff as given by the following equation: ED_{50}/puff = (35 mL of smoke/puff)/(number of mL of smoke/ED_{50}). The smaller the value, the less biologically active is the smoke in the given assay.

As with the mouse skin painting experiments, various statistical tests were used to correlate the smoke chemistry results with those of the bioassays. Hydrogen cyanide and formaldehyde were strongly correlated with increased ciliatoxicity. Formaldehyde, weak acids, very weak acids (but not colorimetric phenols), hydrogen cyanide, TPM, acrolein, and particulate-phase water were significantly correlated with increased cytotoxicity. Isoprene, carbon monoxide, and carbon dioxide were correlated with decreased macrophage inhibition. Phenols (but not very weak acids) were correlated with increased macrophage inhibition. No statistical tests were apparently performed to account for possible second-order terms in the regression equations. In addition, there was apparently no attempt to correlate the results of the mouse skin painting assays with the results of the assays on ciliatoxicity, macrophage inhibition, and cytotoxicity.

TABLE VI

CIGARETTE PARAMETERS FOR TWG STUDY ON EXPERIMENTAL FILTER CIGARETTES

DATA ADAPTED FROM REFERENCE 25

Cigarette Code	TWG III Code	Filter Type	Filter Description	NFDS Removal Efficiency (%)	TPM (mg/cig)	Water (mg/cig)	Nicotine (mg/cig)	PMWNF (mg/cig)	CO (mg/cig)	CO_2 (mg/cig)	Puffs
F1		Control	Empty tube without perforations	-	38.8	8.47	1.67	28.66	15.79	47.94	8.0
F2		Mechanical	Cellulose-untreated	49.3	18.2	2.85	0.81	14.55	18.17	50.73	8.7
F3	91	Mechanical	Cellulose acetate-untreated	48.4	18.5	2.66	0.91	14.92	19.04	52.60	8.5
F4		Charcoal	Cellulose-charcoal-cellulose	44.6	20.7	3.36	1.04	16.31	16.19	46.23	9.0
F5	92	Oxidizing	Cellulose-MnO_4/Al_2O_3-cellulose	46.5	22.5	5.70	1.25	15.52	14.47	46.66	8.2
F6		Acid affinity	Cellulose + 15% polyolimine + 10% $KHCO_3$	51.2	17.7	1.86	1.18	14.64	15.99	47.36	8.8
F7		Acid affinity	Cellulose + 10% $KHCO_3$	45.3	19.8	2.80	1.22	15.82	16.48	47.87	8.6
F8		Acid affinity	Cellulose + 10% polyol + 10% $KHCO_3$	46.2	19.5	2.79	1.17	15.54	16.54	51.68	8.6
F9		Base Affinity	Cellulose + 10% glycerine + 10% citric acid	45.9	19.0	2.31	0.84	15.89	16.03	50.13	8.8
F10		Phenol	Cellulose acetate + 5% triacetin + 5% PEG	48.3	18.7	2.72	0.94	15.04	14.59	50.64	8.4
F11	89	Dilution	Empty tube with perforations	34.2	23.7	3.48	1.36	18.85	7.94	28.13	9.9
F12		Mechanical-Selective	Cellulose-magnesium silicate-cellulose	50.2	16.9	1.80	0.87	14.25	15.60	46.48	8.4

TABLE VII

CIGARETTE PARAMETERS FOR TWG STUDY ON EXPERIMENTAL FILTER CIGARETTES

DATA ADAPTED FROM REFERENCE 25

Code	Filter Type	Formal-dehyde (ug/puff)	Acetal-dehyde (ug/puff)	Acrolein (ug/puff)	Isoprene (relative)	HCN (ug/puff)	NOx (ug/puff)	Colori-metric Phenols (ug/puff)	Very Weak Acids (meq/puff)	Weak Acids (meq/puff)	Cilia-toxicity ED_{50}/Puff	Macro-phage Inhibition ED_{50}/Puff	Cyto-toxicity ED_{50}/Puff	Over-all Rank
F1	Control	4.24	136	47.5	0.10	49.5	53.2	19.22	0.0036	0.0052	0.59	9.2	40.0	12
F2	Mechanical	3.20	129	43.6	0.10	38.0	52.5	8.31	0.0018	0.0029	0.54	6.7	27.5	10
F3	Mechanical	3.16	82	36.6	0.10	31.3	48.3	6.36	0.0021	0.0027	0.43	5.6	25.6	7
F4	Charcoal	1.87	77	13.3	0.05	11.0	64.2	8.15	0.0017	0.0022	0.13	6.2	15.9	2
F5	Oxidizing	2.29	90	24.7	0.07	19.0	20.3	7.63	0.0019	0.0020	0.20	5.8	19.1	4
F6	Acid affinity	1.72	81	29.4	0.10	5.2	50.2	6.93	0.0018	0.0021	0.01	8.3	18.2	5
F7	Acid affinity	2.65	125	38.9	0.11	19.0	51.8	8.28	0.0018	0.0023	0.15	5.8	17.8	3
F8	Acid affinity	2.36	138	34.3	0.12	7.8	57.3	4.31	0.0018	0.0025	0.09	5.6	19.3	1
F9	Base Affinity	3.90	126	41.9	0.10	39.6	41.8	8.96	0.0021	0.0031	0.33	6.2	22.9	8
F10	Phenol	3.75	134	37.3	0.10	32.9	56.6	2.81	0.0018	0.0023	0.44	6.9	30.4	11
F11	Dilution	2.35	54	13.4	0.04	19.7	22.3	12.98	0.0022	0.0028	0.10	13.5	20.9	9
F12	Mechanical-Selective		90	14.6	0.11	38.3	55.6	7.16	0.0014	0.0022	0.40	6.2	17.5	6

Some other studies that were done at around the same time as the TWG were those in support of Cytrel, a cellulose-based synthetic smoking material developed and extensively tested by Celanese (55, 56, 57, 58, 59, 60, 61). While smoke chemistry data can be found in Reference 55, more detailed information can be found in several papers published in Beiträge (62, 63, 64). Cytrel was included in the TWG studies and was given the designation of ATS-B. It was included in several sets of experimental cigarettes as the results obtained were different than expected. In each case, the condensates from Cytrel cigarettes were more tumorigenic than those from the SEB cigarettes (21, 22, 23). On the other hand, Bernfeld and Homburger conducted four studies from 1964 through 1971 and came to different conclusions. They were 1) on a weight/weight basis SC (smoke condensate) from the Cytrel products tested was no more tumorigenic and, in most cases, even less so than that of cigarette tobacco; 2) higher doses of SC from the Cytrel variants are required to cause a biologic response (benign and malignant skin lesions) equal to that of cigarette tobacco; and 3) comparison of SC from 1:1 blends of Cytrel and cigarette tobacco with that of the tobacco alone proved the blend to be less tumorigenic than tobacco alone at two lower dose levels; however, at a higher dose level they were about equal in biologic activity, but in no instance was there any evidence for synergism between the two components of the blend (61). An additional discussion of Cytrel will be found later in this paper in the section dealing with the correlation of smoke chemistry with biological activity.

Epidemiology

The recent Institute of Medicine Report, "Clearing the Smoke," put emphasis on the use of epidemiological results as possibly the best predictor of whether or not a new type of tobacco product, which has been designed to reduce health risks, truly has reduced health risks when compared with conventional products. Now, if chemistry is to be useful, chemistry results

should be able to predict the outcomes of such epidemiological studies. However, such studies may only be valid if they monitor the health outcomes over years of use. Apparently, no such studies have been done with cigarette products that are designed to be in the words of the IOM, "Potentially Reduced Exposure Products," or PREPS (4). However, there is more than enough data to see how chemistry results correlate with other biological data; and that topic will be covered later.

Epidemiology related to tobacco products does show its power to differentiate among tobacco products in three areas: 1) conventional wet snuff versus Swedish snus (a type of wet snuff); 2) cigarettes made from dark air-cured (black) tobaccos versus cigarettes made from blond (flue-cured, Virginia) tobaccos; and 3) charcoal-filtered cigarettes versus those with conventional cellulose acetate filters.

The epidemiology of Swedish snus has been studied by many researchers (65, 66, 67, 68, 69, 70, 71). One conclusion that can be taken from these epidemiological studies is that Swedish snus apparently does not have the health risks that have been ascribed to wet snuff that has been manufactured in the United States (72, 73, 74, 75, 76, 77) and other countries (78).

Hoffmann and coworkers reported on the potential toxic agents in smokeless tobacco products (74, 79, 80). Comparisons of various smokeless tobacco products are shown in Table VIII. According to Nilsson (68), the dissimilarities in the TSNA contents of oral snuff products may be the most important reason for the different outcomes of the epidemiological studies conducted in the United States and Sweden. Recently, Rodu and Cole reported that users of dry snuff had a higher incidence of oral cancer than did users of wet snuff (81). Brunnemann and coworkers reported in 1987 that the total TSNA levels in three brands of dry snuff ranged from 37 to 135 μg/g based on wet weights while those for the five brands of moist snuff products ranged from 5 to 151 μg/g based on wet weights (82).

TABLE VIII

COMPARISON OF SWEDISH SNUS WITH OTHER SMOKELESS TOBACCO PRODUCTS

SELECTED ANALYTES ON A DRY-WEIGHT BASIS

Analyte	GothiaTek® Snus (Maximum Levels) Ref. 83	Moist Snuff (Range. 5 brands) Ref. 79	Moist Snuff (Range. 5 brands) Ref. 80	Moist Snuff (Range. 5 brands) Ref. 74
Nitrite (ppm)	7	NR	NR	2 - 163
Total TSNA (ppm)	10	10 - 289	10 - 288	5 - 26
NDMA (ppb)	10	4 - 102	14 - 67	NR
B[a]P (ppb)	20	<0.1 - 63	<0.1 - 63	NR
Cadmium (ppm)	1	NR	0.45 – 1.58	NR
Lead (ppm)	2	NR	0.86 – 2.96	NR
Arsenic	0.5	NR	NR	NR
Nickel	4.5	NR	NR	NR
Chromium	1.5	NR	NR	NR

The second example where epidemiology has made a clear distinction between types of tobacco products concerns cigarettes made from dark-air-cured (black) tobaccos. Epidemiological studies have shown that smokers of dark tobacco cigarettes have higher risks for smoking-related diseases than did smokers of blond cigarettes (84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94). The results of the studies dealing with lung cancer and dark tobacco cigarettes have been recently summarized (95). That summary reported that smokers of black (dark air-cured) tobacco cigarettes have about 1.75 times the risk of lung cancer than do smokers of only blond (light) tobacco cigarettes. In addition to the epidemiological findings, condensate from black tobacco cigarettes had more activity than condensate from blond cigarettes in mouse skin

painting experiments (96). The condensate from black tobacco cigarettes was found to be more mutagenic than the condensate from blond cigarettes (97).

Why are dark air-cured cigarettes different from American-blend or Virginia style cigarettes? The first clue comes from the routine analyses of the tobaccos. The traditional black tobacco cigarettes have been reported as having low or no detectable reducing sugars (98). Very high concentrations of tobacco specific nitrosamines (TSNA) have been reported in dark tobacco cigarettes, which were high in nitrate (99). Table IX shows data on various dark air-cured, blended, and Virginia style filter cigarettes from the early 1970s (100). These data show the very low sugar content of the air-cured products relative to the other products shown in the table. Also, the air-cured products appeared to have higher blend magnesium levels. Table X shows the smoke data for the same cigarettes. The analytes that were measured at that time do not clearly distinguish among the various types of tobaccos except that the levels of smoke phenols appeared to be higher in the air-cured products as opposed to the other types of products.

Table XI shows routine cigarette and smoke data for three styles of contemporary dark air-cured products. These data were provided by Dr. J. Sarabia of Altadis. Those familiar with cigarette design will note that relatively low amounts of filter ventilation were needed to achieve the given deliveries relative to those needed for American-blend products. Another noticeable difference with American-blend products is the high carbon monoxide deliveries relative to TPM.

Table XII shows more detailed tobacco and cigarette data for two international blended products (Blended 1 and Blended 2) and a European dark air-cured product. All three products were nonventilated filter kings. These data were generated by my colleagues here at Brown & Williamson Tobacco on cigarettes that were purchased in international markets earlier this year. Relative to the two blended products, the tobacco in the dark-air-cured product showed values for humectants, sugars, and polyphenols (chlorogenic acid, rutin, and scopoletin) that were below the lower limits of quantitation for our methods. The level of alkaloids was lower than that

generally found in blended products. In addition, the tobacco from the dark-air-cured cigarette was higher in ammonia, chlorides, and nitrates. The tobacco pH was over 6.6. While the tobacco chemistry for the dark-air-cured product is atypical of blended product, the cigarette parameters are well within the values expected for blended products.

Table XIII shows smoke chemistry data for the same three products. Data were obtained under ISO smoking conditions. While not shown in this Table, the TPM delivery for Blended 1 was about 19 mg/cigarette while the TPM-levels for Blended 2 and the dark-air-cured product were about 14 mg/cigarette. There was a limited supply of these cigarettes so routine smoke chemistry data were not obtained. Also, not all analytes were determined that would have been determined if more product had been available.

The left side of the table shows the deliveries of those analytes whose values are given in terms of μg/cigarette. This includes the vapor-phase Hoffmann analytes and the phenolics. The right side of the table shows the deliveries of those analytes whose values are given in terms of ng/cigarette. In terms of the vapor-phase analytes and the phenolics, there were few analytes that distinguish among the three products except for the reduction in aldehydes, and the increase in phenol. The reduction in formaldehyde has been shown to be a consequence of tobacco and smoke ammonia (101). This also may be the reason for the reduction of other aldehydes relative to the other two products. The reason for the increase in phenol cannot be ascertained from the available data. There is a clear distinction between the blended product and the dark-air-cured product in terms of the PAHs and aromatic amines. In particular, the levels of 1-amino- and 2-aminonaphthalene and 3-amino- and 4-aminobiphenyl were much higher in the dark-air-cured product than they were in the blended product even though the TPM deliveries of the two products were similar. This difference in aromatic-amine deliveries has been shown to be reflective in urine mutagenicity (102). It has been postulated as a reason for the higher incidence of bladder cancer among smokers of dark-air-cured cigarettes (102, 103). The elevations in lung cancer with such cigarettes have been ascribed to NNK levels (92).

The third example of where epidemiology appears to have distinguished between tobacco products is based on the comparison of smoking and lung cancer risks in American and Japanese men (104). Djordjevic and co-workers reported that on an equi-nicotine basis, Japanese cigarettes deliver significantly less "tar" constituents including B[a]P and TSNA (105). Smoke chemistry data from their report is summarized in Table XIV. The authors stated that a statistical analysis of their data showed that there was no significant differences in the comparisons between the U.S. products and similar Japanese products for TPM, "tar", and carbon monoxide. On the other hand, they stated that there were statistically significant differences (U.S. products > Japanese products, $p < 0.05$) for NAT, NAB, NNN, NNK, and B[a]P. Unfortunately, Djordjevic and coworkers did not report on any of the vapor-phase components that would have been expected to be reduced in the charcoal filtered products available in Japan or comment on the possible correlation that the reduced vapor-phase deliveries might have had with the differences in epidemiology.

TABLE IX

BLEND CHEMISTRIES FOR VARIOUS TYPES OF CIGARETTES IN 1972

ADAPTED FROM REFERENCE 100

Brand	Country of Origin	Blend Type	Ash (%)	PETEX (%)	Nicotine Alkaloids (%)	pH	Reducing Sugars (%)	Total Sugars (%)	Nitrogen (%)	Nitrate (%)	K (%)	Cl (%)	Mg (%)
BOULE NATIONALE	Belgium	Light air-cured	21.2	4.5	1.46	6.0	*	*	3.50	1.46	5.34	1.21	0.76
Gauloise Caporal	France	Dark air-cured	21.6	4.3	1.34	6.1	**	**	3.57	1.59	4.60	1.18	0.74
HB	Germany	German blended	17.7	4.3	1.34	5.7	10.5	2.5	2.50	1.59	3.95	0.53	0.52
Hi-Lite	Japan	Modified Virginia	16.6	3.5	1.78	5.4	13.2	18.0	2.03	0.58	4.10	1.27	0.41
Johnson	Belgium	Dark air-cured	21.4	5.0	1.62	6.2	**	**	3.85	1.42	5.63	1.19	0.83
KOOL	USA	US Blended	17.3	5.4	2.02	5.5	3.0	8.9	2.90	1.64	4.25	0.75	0.52
Marlboro	Switzerland	US Blended	16.9	5.4	1.98	5.6	8.3	11.3	2.98	1.42	3.60	0.72	0.53
Marlboro	USA	US Blended	16.9	4.9	1.92	5.7	7.0	10.1	3.20	1.73	3.90	0.78	0.53
MARY LONG	Switzerland	Medium air-cured	22.0	4.2	1.76	5.7	*	5.0	2.61	1.33	5.94	0.80	0.57
Players No. 6	UK	English Virginia	13.1	5.6	2.08	5.4	11.9	17.4	2.30	0.40	3.17	0.87	0.48
PRIMEROS	Switzerland	Medium air-cured	21.4	3.7	1.29	5.6	**	**	3.27	1.90	5.24	0.82	0.68
RIVIERA	Mexico	Sun-cured	17.3	3.3	1.12	5.3	10.8	11.5	2.45	0.84	4.08	0.91	0.56
St. Michel	Belgium	Dark air-cured	20.6	5.1	1.17	6.3	*	*	3.11	1.28	4.39	0.80	0.86
VICEROY	USA	US Blended	17.3	5.0	1.83	5.3	9.7	11.3	2.76	1.68	4.22	0.84	0.51

* Results between 2.0 and 5.0
** Results less than 2.0

TABLE X

SMOKE CHEMISTRIES FOR VARIOUS TYPES OF CIGARETTES IN 1972

ADAPTED FROM REFERENCE 100

Brand	Country of Origin	Blend Type	TPM (mg/cig)	TPM/puff	Total Nicotine Alkaloids (mg/cig)	Phenols (μg/cig)	Puffs	Smoulder Rate (mm/min)	CO (mg/cig)
BOULE NATIONALE	Belgium	Light air-cured	26	2.95	1.39	97	8.8	3.5	22.9
Gauloise Caporal	France	Dark air-cured	28	3.01	1.61	155	9.3	3.1	23.4
HB	Germany	German blended	20	2.15	0.88	53	9.3	4.9	16.8
Hi-Lite	Japan	Modified Virginia	33	3.40	1.81	90	9.7	4.1	21.7
Johnson	Belgium	Dark air-cured	24	3.28	1.54	119	7.3	4.8	20.9
KOOL	USA	US Blended	23	2.60	1.52	42	8.2	5.2	NR
Marlboro	Switzerland	US Blended	31	3.10	1.89	81	10.3	4.1	NR
Marlboro	USA	US Blended	24	2.63	1.42	58	10	4.9	15.9
MARY LONG	Switzerland	Medium air-cured	25	2.87	1.11	70	8.7	4.2	19.1
Players No. 6	UK	English Virginia	24	3.20	1.33	92	7.5	3.9	NR
PRIMEROS	Switzerland	Medium air-cured	31	3.92	1.24	59	7.9	3.9	19.1
RIVIERA	Mexico	Sun-cured	40	3.57	1.33	95	11.2	4.5	34.2
St. Michel	Belgium	Dark air-cured	34	3.36	1.60	113	10.1	3.4	18.9
VICEROY	USA	US Blended	21	2.80	1.28	47	7.5	3.9	18.4

NR = Not reported

TABLE XI

ROUTINE CIGARETTE AND SMOKE DATA FOR CONTEMPORARY DARK-AIR-CURED CIGARETTES

DATA FROM J. SARABIA - ALTADIS

Style	Tobacco Weight (mg)	Filter Ventilation (%)	Nicotine Alkaloids (%)	Reducing Sugars (%)	TPM (mg/cig.)	TPM/Puff	Total Nicotine Alkaloids (mg/cig.)	PMWNF (mg/cig.)	Puffs	CO (mg/cig.)
Ultralight KS	664	29	1.57	0.0	5.1	0.8	0.29	4.2	6.3	9.4
Light KS	707	12	1.49	0.0	9.7	1.4	0.53	7.6	7.0	12.6
Full Flavor KS	692	NR	1.45	0.0	13.1	2.0	0.61	10.3	6.7	14.8

TABLE XII

TOBACCO AND CIGARETTE DATA FOR BLENDED AND DARK-AIR-CURED CIGARETTES

Parameter	Blended 1	Dark-Air-Cured	Blended 2
Ammonia (%)	0.13	0.39	0.15
Theobromine (%)	BLOQ	BLOQ	BLOQ
Glycyrrhizic acid (%)	BLOQ	BLOQ	0.02
Glycerine (%)	3.07	BLOQ	1.81
Propylene Glycol (%)	BLOQ	BLOQ	0.65
Chlorogenic Acid (%)	0.53	BLOQ	0.56
Rutin (%)	0.45	BLOQ	0.47
Scopoletin (%)	0.03	BLOQ	0.03
Alkaloids (%)	2.14	1.59	2.33
Chlorides (%)	0.88	1.68	0.70
Fructose (%)	2.75	BLOQ	3.31
Glucose (%)	1.45	BLOQ	2.34
Nitrates (%)	1.01	1.67	0.88
Phosphates (%)	0.77	0.80	0.60
Sucrose (%)	0.34	BLOQ	3.34
Reducing Sugars (%)	4.20	BLOQ	5.65
Total Sugars (%)	4.54	BLOQ	8.99
Tobacco pH	5.38	6.66	5.56

Parameter	Blended 1	Dark-Air-Cured	Blended 2
Cigarette Pressure Drop (in. water)	5.09	4.36	5.41
Tip Ventilation (%)	0.1	0.6	0.3
Circumference (mm)	24.6	25.1	24.9
Length Cigarette (mm)	83.6	83.6	83.6
Length Filter (mm)	22.3	21	20
Length Tipping (mm)	26	25	24
Length Tobacco Section. (mm)	61.3	62.6	63.6
Cigarette Weight (mg)	1022	924	925
Tobacco Weight (mg)	808	717	730
Density (mg/cc)	273	229	233
Nontobacco Weight (mg)	214	207	195
Filter Weight (mg)	124	112	109
Filter Triacetin (%)	7.99	8.27	6.94
Paper Porosity (CU)	45	45.3	42.2
Paper Citrate (%)	0.65	0.51	1.93

BLOQ = Below lower limit of quantitation

TABLE XIII

SMOKE CHEMISTRY DATA FOR BLENDED AND DARK-AIR-CURED CIGARETTE

Analyte (μg/cig)	Blended 1	Dark-Air-Cured	Blended 2
Hydrogen sulfide	38.3	25.9	24.8
Hydrogen cyanide	71.7	45.5	49.1
Methanol	138	23.9	115
Formaldehyde	40.8	21.2	39.6
Acetaldehyde	598	424	520
1,3-Butadiene	28.9	22.9	21.1
Acetonitrile	140	123	99.5
Acrolein	54.2	33.7	45.2
Furan	22.2	14.6	18.1
Propanal	35.0	22.5	28.7
Acetone	245	186	198
Acrylonitrile	10.5	8.6	8.2
Carbon disulfide	3.4	2.8	2.5
Isoprene	187	119	160
Propionitrile	23.2	20.2	18.1
2-Methylpropanal	10.1	7.0	7.5
Methylethyl ketone + Butanal	66.4	42.8	54.5
Crotonaldehyde	6.5	5.1	6.0
Benzene	29.6	28.5	27.4
Pyridine	1.2	1.0	1.0
Toluene	18.8	20.2	17.0
Styrene	1.1	1.2	1.1
Phenol	16.4	23.0	13.5
o-Cresol	4.6	6.3	3.9
m-Cresol	3.7	4.7	3.2
p-Cresol	8.6	13.5	6.9
Catechol	56.1	40.3	48.4
Resorcinol	1.1	0.8	1.3
Hydroquinone	51.7	30.4	52.8

Analyte (ng/cig)	Blended 1	Dark-Air-Cured	Blended 2
Naphthalene	936	1088	632
Fluorene	350	358	232
Phenanthrene	230	258	171
Anthracene	78.4	75.2	47.2
Fluoranthene	95.2	102	69.6
Pyrene	67.2	64.4	46.4
Benzofluorene	40.8	41.6	25.8
Benzanthracene	22.9	24.7	13.8
Chrysene	26.7	24.6	18.2
Benzofluoranthene	18.9	16.8	12.3
Benzo[e]pyrene	9.1	7.9	5.7
Benzo[a]pyrene	11.5	9.8	7.8
Perylene	1.6	1.1	1.0
Dibenzanthracene	0.6	0.6	0.3
Benzoperylene	3.8	2.6	2.4
Aniline	NA	640	408
o-Toluidine	NA	209	118
m-Toluidine	NA	294	181
p-Toluidine	NA	271	150
2-Ethylaniline	NA	64.0	37.4
2,6-Dimethylaniline	NA	6.16	2.88
2,5-Dimethylaniline	NA	64.8	39.46
2,4-Dimethylaniline	NA	60.4	31.8
3-Ethylaniline	NA	68.4	47.2
3,5-Dimethylaniline	NA	43.6	25.7
2,3-Dimethylaniline	NA	9.92	5.24
4-Ethylaniline	NA	93.2	54.8
3,4-Dimethylaniline	NA	43.6	25.8
1-Aminonaphthalene	NA	75.6	38.8
2-Aminobiphenyl	NA	10.1	5.12
2-Aminonaphthalene	NA	141	69.6
3-Aminobiphenyl	NA	10.6	5.52
4-Aminobiphenyl	NA	6.56	3.88
Benzidine	NA	0.57	0.59

TABLE XIV

SMOKE CHEMISTRY DATA FOR U.S. AND COMPARABLE JAPANESE PRODUCTS

DATA ADAPTED FROM REFERENCE 105

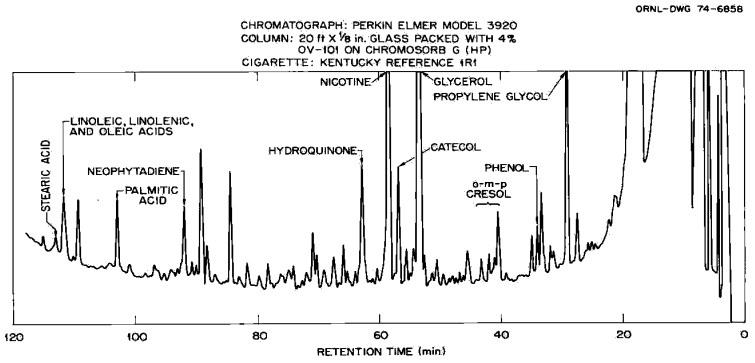
	TPM	"Tar"	Nicotine	CO	NAT	NAB	NNN	NNK	Total TSNA	B(a)P
U.S. Brands	(mg/cig.)	(mg/cig.)	(mg/cig.)	(mg/cig.)	(ng/cig.)	(ng/cig.)	(ng/cig.)	(ng/cig.)	(ng/cig.)	(ng/cig.)
KS, F, SP, LT	12	10	0.8	10	183	18	165	117	483	11.1
KS, F, SP, LT	10	9	0.8	10	136	17	146	98	397	13.5
KS, F, SP, LT, MEN	11	10	0.8	12	145	12	145	92	394	9.2
KS, F, SP, LT, MEN	10	9	0.7	11	187	27	210	144	568	11.2
KS, F, SP	21	16	1.1	14	251	23	256	145	675	14.2
KS, F, SP, MEN	20	16	1.1	15	312	35	297	106	750	11.2
KS, F, SP (charcoal)	16	15	1.0	15	200	24	219	166	609	9.3
KS, F, SP	20	17	1.4	14	249	26	236	216	727	11.6
Japanese Brands										
KS, F, SP, LT	8	6	0.7	6	62	8	36	37	143	5.1
KS, F, SP, LT	8	7	0.6	6	109	13	75	49	246	5.8
KS, F, SP, LT	11	10	1.0	8	96	11	70	38	215	8.1
KS, F, SP	16	12	1.2	13	130	13	96	66	305	9.9
KS, F, HP	13	10	1.1	8	138	21	129	58	346	8.8
KS, F, SP	21	16	1.6	19	87	12	64	53	216	13.3

Correlations among smoke chemistry and measures of smoke toxicity

It is relatively easy to generate large quantities of data on the mainstream smoke from various brands of cigarettes. Indeed, if one has the financial resources, commercial laboratories that do the job for you and present the data neatly tabulated in electronic spreadsheets or other common formats for electronic data. Since data from the laboratories that provide *in vitro* or *in vivo* also can provide data electronically, it would appear that making correlations between smoke chemistry and biological effects is a simple matter of mixing and matching data with one's favorite statistical analysis package. Obviously, this is not the case; and this has been a historical problem. Data presentation also is a problem when discussing the results of various studies with those who may be less familiar with the details of the chemistry and toxicological assays. One of the early approaches to the problem was developed by Guerin and Nettesheim (32).

Figure 5, which was taken from Reference 32, shows the resolution of the chromatograms for silylated mainstream TPM at the time of the TWG. This chromatogram was obtained with a packed column, and t much more complicated chromatograms can be

Figure 5



obtained with today's fused silica capillary columns. However, the numerous peaks in this chromatogram are sufficient to document the challenge of correlating chemical data with the results from *in vitro* and *in vivo* toxicological assays. Now, if one is lucky, one may get a

reasonable linear correlation with one or more of the peaks in the chromatogram and one or more toxicological endpoints. An example of this is shown in Figure 6, which was taken from Reference 32.

Figure 6

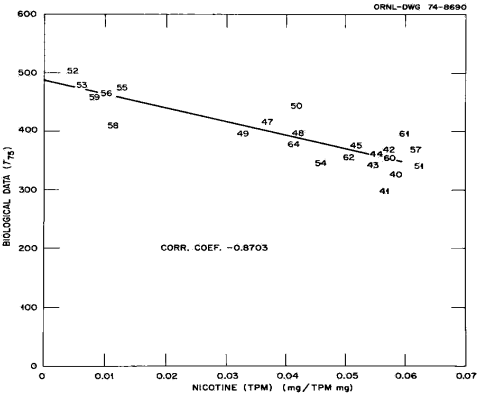
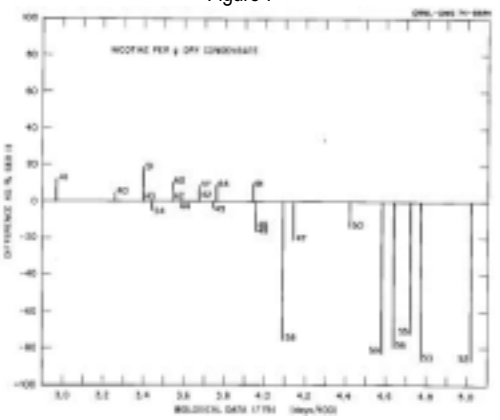


Figure 6 shows the biological response from a mouse skin painting study (T_{75}, which is the estimated number of days from the initiation of the experiment that 75% of the mice would survive) with the concentration of nicotine in the TPM. Another way of presenting the same data is shown in Figure 7, which shows the results versus the reference product (SEB II) (32).

Figure 7



Since the mouse skin painting assays are usually done on a per unit weight of condensate basis, there is no correction for the differences in deliveries among the cigarettes in the study (i.e., nonfilter high-delivery products would be considered on same basis as ultralight products). This is a very important consideration because of the partitioning of the semivolatile components of the TPM between the vapor and particulate phases of the smoke aerosol (106, 107). If the smoke collection system used to trap the TPM allows loss of part of the semivolatiles, then the TPM becomes enriched in the nonvolatile materials. The nonvolatile components of TPM are believed to be responsible for much of the biological activity associated with the particulate phase of the smoke aerosol (5, 6, 7, 11, 108, 109, 110, 111). This may be one reason that condensate mutagenicity (TA98+S9, TA100+S9) generally increases as filter ventilation increases when condensate is trapped on the Cambridge pad (112, 113). However, increasing puff volume and puff frequency decreases condensate mutagenicity (114). Chortyk and Chamberlain reported a decrease in Ames activity with a decrease in mainstream deliveries when the smoke condensates were collected in solution (115).

Cytotoxicity studies have become more popular as more laboratories have implemented the Neutral Red Uptake assay (116). This assay has been implemented for whole smoke (117). It also has been implemented for smoke fractions such as TPM, the gas-vapor phase (GVP) passing through the Cambridge pad, and mixtures of GVP and TPM (118, 119, 120, 121). In one sense, the Neutral Red Uptake assay is a modern version of the cytotoxicity assay employed in the TWG studies, and one might expect somewhat similar findings.

In terms of the cytotoxicity of TPM, Bombick and coworkers reported in 1998 that TPM from all flue-cured cigarettes was more cytotoxic than that from all burley cigarettes and that upper stalk tobacco of both varieties gave more cytotoxic condensates than did the corresponding lower stalk tobaccos (122). The same research group has also reported that the cytotoxicities of the condensates from the Kentucky KY1R4F and KY1R5F are representative of those of the light and ultralight “tar” categories, respectively, on the domestic market (123). In

that study, the average EC_{50} values reported for the full flavor, lights, and ultralights groups were 44.8 μg/mL, 51.7 μg/mL, and 38.5 μg/mL, respectively. The values reported for the KY1R4F and KY1R5F were 57.9 μg/mL and 42.5 μg/mL, respectively. It is important to note that the higher the EC_{50} value the less toxic the condensate. Similar findings were reported by Tewes and coworkers although they used a different cell line in their assays (120). They found that cytotoxicities of the condensates from American blend cigarettes differed little from cytotoxicity of the condensate of the KY1R4F. Cytotoxicities of the condensates from single-grade cigarettes were higher than those of the blended products with bright tobacco giving more cytotoxic condensate than burley tobacco. There does not appear to be an explanation for the trends that have been observed nor relationships between the cytotoxicity of the condensates from the domestic brands and the smoke chemistry data provided in the same report (123). Some work has been done with pure compounds likely to be in the condensate. Bombick and Doolittle have shown that 4-vinylpyridine and 2-vinylpyridine are much more cytotoxic than pyridine itself (124). Bombick and coworkers also have shown that phenolic constituents in condensate have little impact on cytotoxicity (125).

The situation appears clearer when whole smoke or just the gas-vapor phase of smoke is considered. Acrolein and to a lesser extent formaldehyde have been found to be much more cytotoxic than the other volatile carbonyl compounds found in mainstream smoke (120, 124). Furthermore, cigarettes made with a carbon filter designed to remove such compounds from mainstream smoke had reduced cytotoxicity with respect to a conventionally filtered product (118). This finding is not a new one. In 1970, Battista and Kensler reported similar findings in an *in vivo* study using chicken trachea (126).

Changes smoke chemistry that are reflected in the *in vitro* Neutral Red cytotoxicity assay have been reported to be reflected in changes observed in rodent inhalation experiments. When apparently the same cigarette samples as described in Reference 118 were used in an acute mouse inhalation study, it was found that the cigarette with the carbon filter reduced the

irritancy of the smoke as measured by the concentration of smoke associated with a 50% decrease in respiration rate (RD_{50}) (127).

In a rat inhalation study published in 1980, Lam found that use of a carbon-filtered cigarette versus a conventionally filtered cigarette gave some surprising results when the biological endpoint was loss of epithelial cells in the larynx (128). Lam reported that at equal particulate matter concentrations the carbon-filtered cigarette caused more pathology than did the conventionally filtered product even though the carbon-filtered product gave 73% reductions in the vapor-phase constituents such as HCN, acrolein, and formaldehyde. Lam hypothesized that the reason for the observed effect was that the carbon filter had removed part of the semivolatiles in the particulate phase thus increasing the effective dose of particulate matter. In addition, the data indicated that in the case where a significant difference in pathology was seen, the smoke concentration for the charcoal filtered cigarette was 4.00% while it was 3.50% for the conventionally filtered cigarette. There are no data in that paper on chemical measurements of the test atmospheres although the methodology for such measurements had been developed by the same laboratory (129).

In another rodent inhalation study from the same era, Coggins, Lam, and Morgan reported the results of a chronic inhalation study using cigarettes containing differing levels of Cytrel tobacco supplement (60) in a flue-cured blend typical of commercial U.K. cigarettes. Cytrel inclusion levels were 0, 25, 50, 75, and 100%. Apparently, very similar cigarettes were used as part of the evaluation of the continuous-smoking inhalation machine described in Reference 129, and the smoke deliveries of other Cytrel-containing cigarettes have been published (62, 63, 64). The relevant smoke chemistry and histopathological data are summarized in Tables XV and XVI.

TABLE XV

CYTREL INHALATION STUDY

DATA ADAPTED FROM REFERENCES 60 AND 129

Cytrel inclusion (%)	Average puffs/cig. (per session)	Cigarette consumption (%)	Smoke dilution (%)	PMWNF (mg/m^3)	PMWNF (mg/cig)	Nicotine (mg/cig)	CO (mg/m^3)	CO (mg/cig)	HCN (μg/cig)	Aldehydes (mg/cig)	HCHO (μg/cig)	Phenols (uμg/cig)
0	8.3	248	1.74	550	16.3	1.18	616	16.2	297	1.9	82	87
25	7.4	294	1.75	582	NR	NR	750	NR	NR	NR	NR	NR
50	6.3	325	1.94	479	8.5	0.32	807	10.9	136	1.2	62	33
100	5.3	378	5.41	619	3.1	0	1403	4.5	7	0.2	10	15

TABLE XVI

CYTREL INHALATION STUDY

DATA ADAPTED FROM REFERENCES 60 AND 129

Cytrel inclusion (%)	Body weight male (g)	Body wt female (g)	COHb (%) male	COHb (%) female	Nasal Epithelium (0-3)	Laryngeal Epithelium (0-3)	Tracheal Epithelium (0-3)	Bronchial Epithelium (0--3)	Brown Gold Macrophages (0-3)
0	398	288	26.9	29.9	1.13	1.76	0.73	0.36	1.33
25	415	274	33.0	38.4	1.05	1.50	0.71	0.40	1.27
50	425	266	27.7	34.0	1.10	1.67	0.76	0.46	1.29
100	387	268	43.3	48.8	0.69	1.36	0.38	0.34	0.49
Room Control	652	442	NR	NR	0.27	0.27	0.10	0.03	0.01
Machine Control	506	314	NR	NR	0.16	0.19	0.08	0.04	0.00

While no statistical analyses of the data have been done, one can see that as the percentage of Cytrel in the blend increased, there was a decrease in the analytes associated with irritation and cytotoxicity. The severity of the lesions reported in the histopathological examinations for the nose, larynx, and trachea decreased as the percentage of Cytrel in the blend increased. These differences in pathology became statistically significant between the sample with 50% Cytrel inclusion and the sample that was 100% Cytrel. However, it is not known from the available data as to which, if any, of the measured analytes was responsible for the change in the biological outcomes.

It is clear from the examples in the literature that reductions in some measured analytes in some tests do correlate with reductions in biological potency in certain assays. However, generally technology that results in the reduction of a measured analyte, results in changes of other analytes, measured or not. Indeed, in the case of mainstream cigarette smoke, the unmeasured analytes outnumber the measured analytes by more than 100:1. Therefore, correlation of specific analytes with the results of biological assays is not necessarily proof that the measured analytes were the only ones responsible for biological activity. For example, Battista listed sulfur dioxide and 2,3-butanedione as having the same order of ciliatoxicity as ammonia and crotonaldehyde (130). The former two compounds are not generally measured as part of the Hoffmann analytes, but the latter two are.

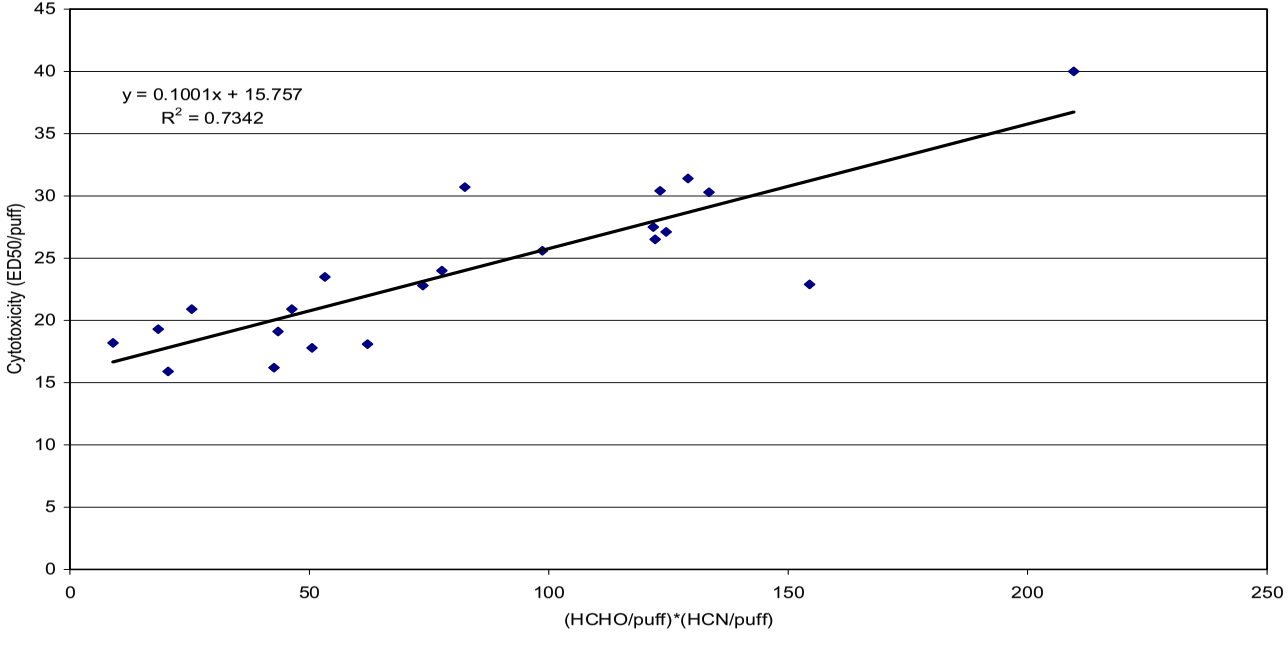
Another factor that tends to limit the usefulness of correlations between smoke chemistry and biological activity is the neglect of nonlinear relationships. First, many biological processes are nonlinear with respect to dose. For example, the dose-response curves for the Neutral Red Uptake assay of mainstream cigarette smoke are best described by a logistic function as reported by Roemer and coworkers (121). Second, there are interactions among the chemical constituents in smoke. One of these, the interaction of HCN and aldehydes, was described by James Nall of the Brown & Williamson Tobacco Corporation at the 20th TCRC in 1966 (131).

Figure 8 shows the relationship between cytotoxicity (EC50/puff) and the product of the per puff deliveries of HCN and formaldehyde for the different cigarettes designs using the straight SEB III blend and casings in TWG-III. Another known case involving the reactivity of formaldehyde that may be important in understanding biological endpoints is its reaction with ammonia (101). While there was consideration and use of second-order and cross-product terms in Bayne's analysis of the TWG mouse skin painting data (34), the significance of some of his terms (i.e., $[pH]^2$) with respect to the correlations between the smoke chemistry and the biological effect remain a matter of speculation. Therefore, correlation of smoke chemistry with biological activity should be a joint effort of chemists, toxicologists, and statisticians.

Recently, two reports detailed the results of surveys of smoke chemistry for a number of brands on the U.S. market. The first of these provided data on both chemistry and mutagenicity (113) while the latter just included smoke chemistry (132). Both reports show numerous graphs detailing the linear relationships (some better than others) of many of the Hoffmann analytes with FTC "tar", and the second report also provides an extensive compilation of smoke chemistry data for numerous cigarette brand styles. While these reports show interesting ways of processing and presenting smoke chemistry data, it is important to note that the purposes of the reports were somewhat different. The purpose of the first report was to show that the KY1R4F and KY1R5F reference cigarettes were acceptable reference cigarettes for comparative mutagenicity and smoke chemistry studies on cigarettes in the U.S. domestic market (113). The purpose of the second report was to show that the smoke constituent yields of commercially marketed cigarettes in the U.S. between 1995 and 2000 have been effectively constant (132).

Another approach to presenting smoke chemistry data has been given by Rustemeier and coworkers as part of Philip Morris's ingredient study (13, 133). They used radar charts to illustrate the differences in analytes from test and control products.

Figure 8
Cytotoxicity (ED50/puff) vs. (HCHO/puff)*(HCN/puff)



SUMMARY

In this report, the complex subject of the relationships among tobacco, tobacco smoke, and a variety of biological endpoints has been covered from three perspectives: 1) a review of the studies by and associated with the Tobacco Working Group (TWG); 2) a review of the three commonly recognized relationships between tobacco or smoke chemistry and the results of epidemiological studies; and 3) a review of studies dealing with relationships between smoke chemistry and various biological endpoints and different strategies for correlating chemistry alone or with biological endpoints.

The TWG, which was essentially a study in PREPS based on conventional product technology, used many of the testing strategies that we use today in assessing the health-related aspects of tobacco products: 1) tobacco and/or smoke chemistry; 2) *in vitro* toxicology; and 3) *in vivo* toxicology. In addition, extensive use of statistics was made to identify the factors responsible for the differences in biological endpoint among the different cigarette designs. The TWG studies also pointed out the danger in using statistics as nicotine was found to be highly correlated with tumorigenicity and very significant efforts were required to show that this correlation was no doubt caused by minor components in smoke that were well correlated with nicotine.

The second part discussed the three cases of where epidemiological results have been correlated with product chemistry. The three cases: 1) Swedish snus versus conventional snuff products; 2) dark-air-cured (black) tobacco cigarettes versus blended and Virginia-style products; 3) Japanese versus U.S. domestic cigarettes show the apparent relationships between product chemistries and some diseases associated with tobacco use. These are apparent relationships and more studies will be needed to confirm the chemistries or other factors that may be responsible for the epidemiological findings.

The final section of this report covers specific examples of where smoke chemistry has been correlated with specific in vitro and/or in vivo endpoints. This section also states the need for both more in-depth chemical studies and/or the use of nonlinear statistical techniques to find better correlations between smoke chemistry and biological endpoints. In particular, results of assays of TPM in the Neutral Red Uptake assay for cytotoxicity do not appear correlated with most chemical measures employed on mainstream cigarette smoke.

In conclusion, the challenges of correlating tobacco and/or smoke chemical data with toxicological data remain after much work by numerous chemists, toxicologists, and statisticians associated within our industry. We now must use advances in analytical chemistry and toxicological sciences to continue the quest for understanding these complex relationships.

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Tobacco Smoke Genotoxicity: An *In Vitro* Perspective

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1. Summary

An overview of the *in vitro* genotoxic activity of tobacco smoke has been set out with a theme to consider what is known about putative components responsible for activity in some key test systems. With the considerable variety of genotoxicity test systems available, those assays featured most commonly in regulatory guidance have been selected namely: 1) Gene mutations and 2) Structural and numerical chromosome changes. For the gene mutations, the Ames bacterial mutagenicity system has been used to track the influence of nitrogenous components, identifying the pyrolysis products of amino acids as exerting a strong influence upon condensate activity in the presence of an exogenous metabolic activating system. Whilst activity in the mammalian cell based *in vitro* micronucleus assay detecting chromosome changes is not dependant upon a metabolic fraction, work to elucidate responsible compounds is lacking. Most work described has been carried out on cigarette smoke condensate using a mixture of bacterial and mammalian cells. However, experimental procedures to examine whole smoke, i.e. vapor and particulate phase are available in both submersion cell culture and with cells maintained at the air liquid interface; these data have also been reviewed. In conclusion, experiments in the area of molecular biology applied to *in vitro* genotoxicology are described and continue to

demonstrate a striving to understand the possible causative chemistry and the nature of lesions induced by tobacco smoke.

2. Introduction

Following studies with mustard gas in the 1940s by Auerbach and colleagues (1, 2) numerous studies were initiated to screen chemicals for mutagenic activity. However, it was not until the pioneering work of E. C. and J. A. Miller in the 1960s that it was recognized that metabolic activation was required for many chemicals to produce cancer in animals (3, 4). This work subsequently led to the development of techniques for metabolic activation *in vitro* and their subsequent refinement and adaptation to other organisms e.g. bacteria by workers such as B. N. Ames (5, 6) in the mid 1970s. Indeed, at this time there was a high correlation, 90%, between carcinogenic and mutagenic activity (7).

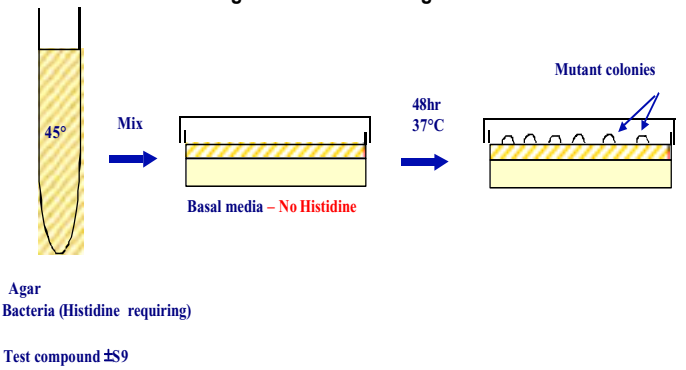
By the early 1980s, more than 40 different 'short term' assays had been proposed for the detection of potential carcinogens (8). This wide variety of tests presented considerable problems for those attempting to recommend a minimum battery of tests to detect the majority of established and potential carcinogens. These concerns led to the beginning of numerous international trials seeking the optimum test battery. Under the auspices of the International Programme on Chemical Safety (8) some 27 assay systems were used to examine the 'activity' of 42 compounds, using where possible, structurally related carcinogen / noncarcinogen pairs. However, even at this time, the bacterial based Ames system was well to the fore as the leading contender for the detection of putative mutagens and carcinogens (5, 6, 7)

and had already been included in early regulatory recommendations (9). In addition, work to examine the effects of smoke condensates in the Ames system had commenced with additional assays in mammalian cells as a recommended complement (10).

3. Ames Tests on Cigarette Smoke Condensate

The Ames test system utilizes *Salmonella typhimurium* that is defective in genes coding for the synthesis of histidine and the end point scored is the number of colonies reverting to histidine auxotrophy. In brief, ~10^8 bacteria are treated with the compound of interest either in the presence or absence of a rat liver supernatant fraction from a liver homogenate following a 9000g centrifugation (S9) Fig. 1. For most testing schedules, the rodent livers are from rats pretreated with compounds such as arochlor 1254 to induce a wide spectrum of xenobiotic metabolizing enzymes. The test bacteria, compound ± S9 and soft agar at 45 °C are mixed and plated out on to basal media containing C, H, O, N and P plus essential minerals and incubated for 48 hours at 37 °C. Bacteria that mutate to histidine auxotrophy by the action of the compound of interest will grow to form individual colonies, which can later be counted (5, 6, 7).

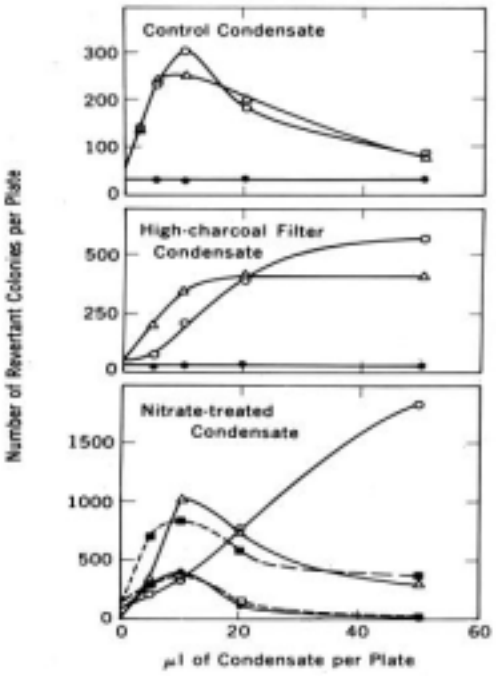
Figure 1. Ames Testing Procedure



Treating with a range of doses will also produce a dose response curve. With two specific Ames strains, TA1538 and TA1535 that detect frameshift and base pair substitution mutagens respectively, activity from cigarette smoke condensates (CSC) was detected in the presence but not in the absence of S9 metabolic activation, (Fig. 2) (10).

It is of interest to note that addition of 10% magnesium nitrate to the tobacco changed the pattern of activity from that of the control untreated tobacco. Activity was now recorded in both the presence and absence of the S9 metabolic activating fraction and was of greater magnitude than that of condensate from the control tobacco (Fig. 2). Activity was also greater with the TA1538 strain, which detects frame shift mutagens in contrast to the base pair substitution sensitive strain TA1535, (Fig. 2).

Figure 2. Ames Mutagenic Activity of Tobacco Smoke Condensates (10)



TA1538 no S9 mix

TA1538 + S9

△ **TA1538 + lung S9**

TA1535 no S9

■ **TA1535 + S9**

This early paper from the Ames group also commenced work to understand

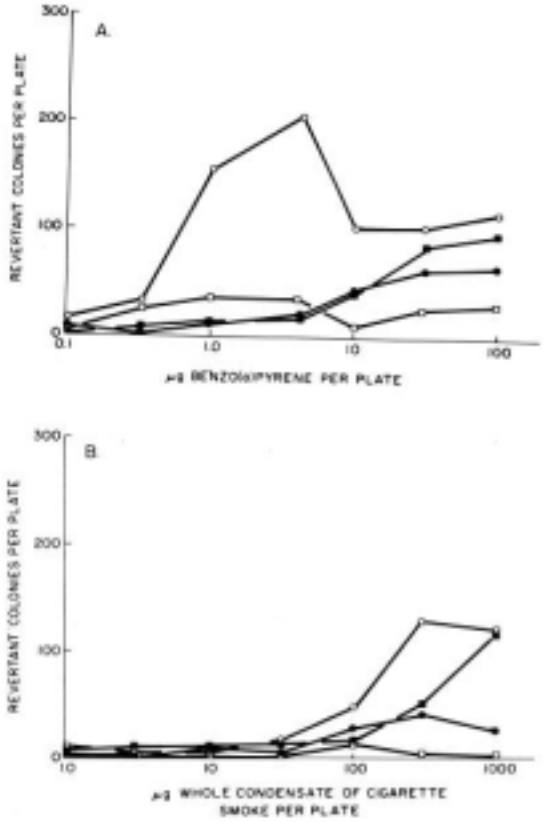
fractions (10). The majority of activity was recorded in the basic fraction with the acid fraction second and minimal activity from the neutral fraction (Table 1).

Table 1. Ames TA1538 Activity of Smoke Condensate Fractions (10)

Fraction	% Activity of whole condensate
Whole condensate	100
Basic	59
Acid	37
Neutral	4
Reconstituted condensate	89

The work on investigating components responsible for the Ames activity of smoke condensates continued with the work of Hutton et al. (11). This group examined the activity of smoke condensate from KY1R4F cigarettes with the *Salmonella* strain most sensitive to smoke condensate, TA1538 and compared this with the activity of benzo(a)pyrene. Tobacco smoke condensate is reported as "much less mutagenic than BP" and from the plots illustrated in Fig. 3, it was estimated that 100 µg of smoke condensate had the same mutagenic activity as 6µg of benzo(a)pyrene. As there was a measured level of 1 µg benzo(a)pyrene per g of condensate, benzo(a)pyrene could only account for a small fraction of the total amount of mutagenic substances in tobacco smoke. This specific paper also reports that nicotine was nonmutagenic in their Ames test system (TA1538+S9) confirming the data of Ames et al. (7, 11).

Figure 3. A Comparison of Ames TA1538 Activity of Smoke Condensate and Benzo(a)pyrene (11)



Untreated rat S9

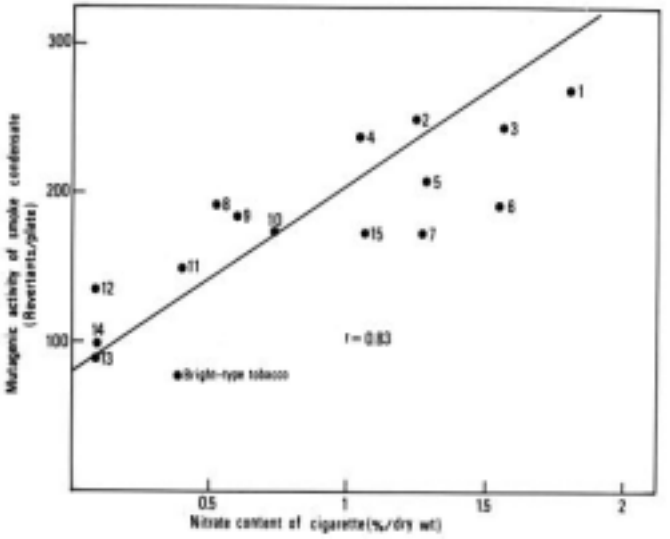
- **Phenobarbitone treated rat S9**

3-methylcholanthrine treated rat S9

Human liver S9

The laboratories of the Japan Tobacco and Salt public Corporation began a significant series of experiments into the Ames bacterial mutagenicity of smoke condensates in the late 1970s (12,13, 14). This group set out to describe the basic mutagenic characteristics of smoke condensates and examine the potential etymology of the recorded activity. In the first paper, a relationship between the nitrate content of the tobacco and the mutagenic activity of smoke condensate (TA1538+S9) was recorded (Fig. 4). These data were further sub divided between Burley and flue-cured tobaccos with activity lower in the flue-cured blends and a positive relationship between the nitrate content of the Burley's and the resultant activity of the smoke condensate (Table 2) (12).

Figure 4. Effects of Tobacco Nitrate Content on the Ames TA1538+S9 Activity (12)



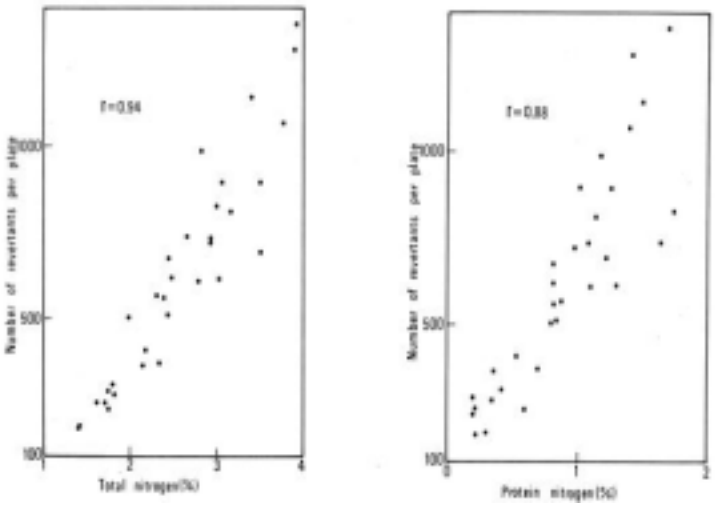
Numbers indicate individual brands tested.

Table 2. Mutagenic Activity of Smoke Condensates from Burley and Flue-cured Tobaccos (12).

Tobacco	Rate of N fertilizer (kg $10a^{-1}$)	Nitrate content % Dry wt.	Revertants 500 ug CSC^{-1} (TA1538+S9)
Burley	9	1.6	162
Burley	13	3.0	227
Burley	18	3.9	270
Burley	25	5.0	293
Flue-cured		0.4	89

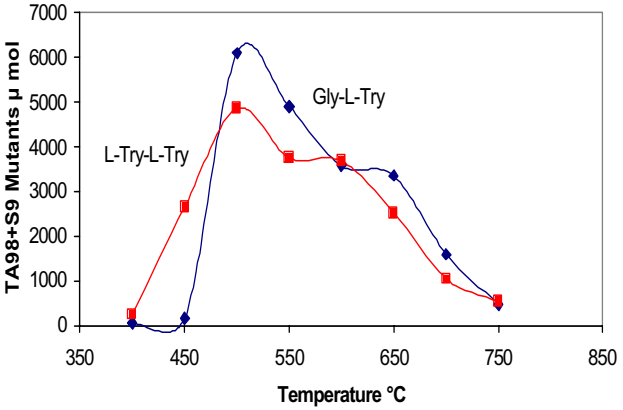
The initial analysis was subsequent refined, correlating the mutagenic activity (TA98+S9) against the total nitrogen content of the tobacco (r=0.94) and the specifically the total protein content of the leaf (r=0.88) (Fig. 5) (13).

Figure 5. Effect of Tobacco Total Nitrogen or Protein Nitrogen on the Ames TA98+S9 activity of smoke condensate (13).



With work in other laboratories on the pyrolysis of amino acids and proteins allied to the work on tobacco, the data suggested a connection between the activity of smoke condensates and protein/amino acid pyrolysis products (14 & 15). The data on two amino acids are illustrated in Fig. 6 showing a temperature dependant activity in the region of temperatures achieved in the burning cigarette (14 & 16).

Figure 6. Mutagenic Activity of Condensates of Glycine (Gly) and L-Tryptophan (L-Try) Pyrolysed at Different Temperatures (14).



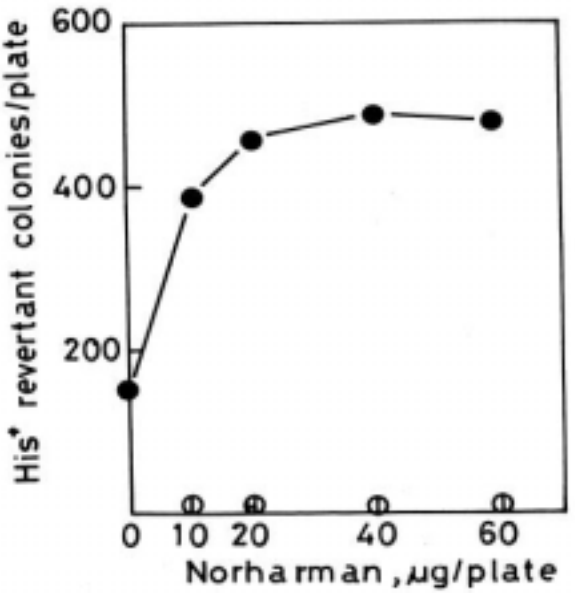
Yoshida and Matsumoto measured the level of potential pyrolysis products in CSC and obtained a positive correlation between the level of amino alpha carbolines and the mutagenic activity of the CSCs (Table 3) (17).

Table 3. Levels of Alpha Amino Carbolines and Mutagenic Activity of Smoke Condensates (17).

Tobacco	Amino alpha carbolines ng cig $^{-1}$	B(a)P ng cig^{-1}	TA98+S9 Mutants cig.$^{-1}$
Flue cured	35	27	126
Burley	132	16	466

This paper also demonstrated that some carbolines such as norharman, which is nonmutagenic, are able to enhance the mutagenic activity of related compounds (Fig. 7) (17).

Figure 7. Ames TA98+S9 Activity of 0.2µg of Amino-α-carboline ( ) in the Presence of Increasing Amounts of Norharman (17).



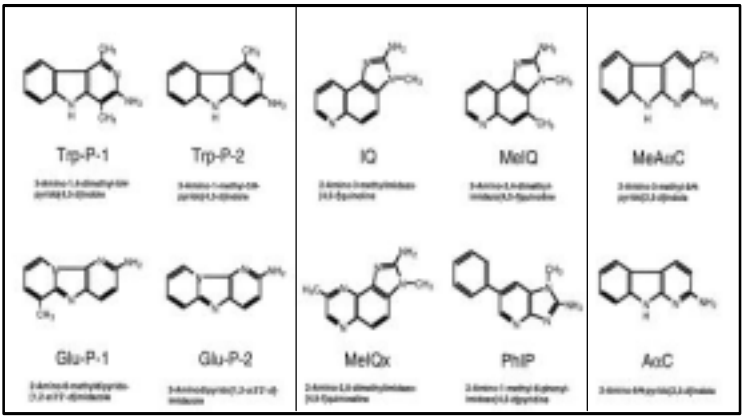
Subsequent work on the activity of a range of pyrolysed materials has revealed the presence of some of the most potent Ames TA98+S9 mutagens to be heterocyclic amines derived from the pyrolysis of amino acids and proteins (Table 4)(18). These can be divided into 2 main types called IQ (2-amino-3-methylimidazo[4,5-f]quinoline) and non-IQ mutagens (Fig. 8). The non-IQ forms are typified by glutamic acid pyrolysate 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) and tryptophan pyrolysate 3-amino-1,4-dimethyl-5Hpyrido[4,3-b]indole (Trp-P-1) and predominate in cigarette smoke condensate (18).

Table 4. Ames mutagenic activity of heterocyclic amines formed during pyrolysis of proteins and amino acids (18).

Chemical	Mutants ug^{-1}	
	TA98 + S9	TA100 + S9
IQ	443000	7000
Glu-P-1	49000	3200
Trp-P-1	39000	1800

Glu-P-1 = 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole
Try-P-1 = 3-amino-1,4-dimethyl-5Hpyrido[4,3-b]indole
IQ = 2-amino-3-methylimidazo[4,5-f]quinoline

Figure 8. Structures of Heterocyclic Amines (32)



Examination of the contribution to the Ames TA98+S9 activity of tobacco smoke condensate has been carried out by the group of Tsuda et al. (19). Known IQ and non-IQ pyrolysis mutagens were subject to either hypochlorite or nitrite treatments (2mM/30min/37 ºC) and the products then Ames tested with TA98 in the presence of S9 (Table 5). The IQ heterocyclic amines are insensitive to nitrite treatment but labile to hypochlorite. In contrast, the non-IQ types are labile to both nitrite and hypochlorite.

Table 5. Treatment of Model Heterocyclic Amines with Nitrite and Hypochlorite (19).

Compound	TA98+S9 % Activity remaining after treatment with:	
	Nitrite	Hypochlorite
Non IQ Types		
Trp-P-1	2	0
Glu-P-1	3	2
IQ Type		
MeIQ	88	0
MeIQx	98	2

Following this proving of the system, condensates of a number of pyrolysates were treated similarly and then Ames tested. These data are illustrated in Table 6.

It can be observed that the majority of mutagens in tobacco smoke condensate are inactivated by the nitrite treatment indicating that 85% the TA98+S9 mutagenic activity is a result of non-IQ type heterocyclic amines. In contrast, the sardine pyrolysate is composed mainly of IQ type heterocyclic amines.

Table 6. Mutagenic Activities of Pyrolysed Materials Following Nitrite or Hypochlorite treatments (19).

Pyrolysate	Treatment			% Mutagenicity due to:	
	None	Nitrite	Hypochlorite	Non-IQ type	IQ type
	Revertants mg basic fraction^{-1}				
Sardine	16000	15600 (97)	1500 (9)	3	88
Tobacco	7280	1123 (15)	680 (9)	85	6

( ) = % residual activity

In reviewing the genotoxic activity of tobacco smoke much work has been centered around guidelines developed by regulatory agencies and protocols have been developed to efficiently detect mutagens using an *in vitro* test

battery. With the Ames system, five strains each with different DNA sequence hotspots used in the presence and absence of an S9 metabolic activating fraction will efficiently detect bacterial point mutagens (37). However, to detect all *in vitro* mutagens the bacterial system should be complemented by assay systems using mammalian cells (20, 21, 22).

4. Assays with Mammalian Cells *In Vitro*

Assays using mammalian cells *in vitro* have centered upon the detection of chromosome aberrations, sister chromatid exchanges (SCE) or the detection of point mutations in mammalian cells. However, in the field of genetic toxicology, emphasis upon SCEs has wained in the late 1990s and the assay is not included in regulatory guidance (20, 21 & 22).

Current, recommendations for *in vitro* genetic toxicology with mammalian cells are for an assay system that is able to detect both structural and numerical changes in chromosomes following treatment with the agent of interest (Fig. 9) (20). This strategy requirement is fulfilled by the *in vitro* micronucleus assay (Fig. 10) (20, 23).

Figure 9. *In Vitro* Screening Tests Recommended by the UK Committee on Mutagenicity

Undertake **Stage 1 tests**

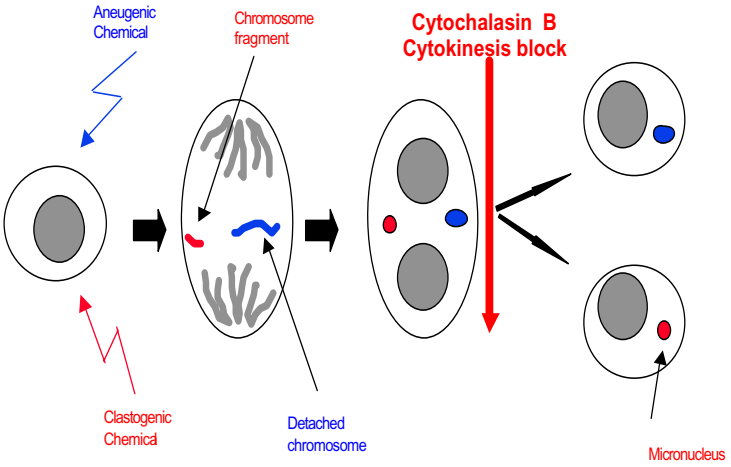
1. Bacterial test for gene mutation
2. Test for clastogenicity and for indications of aneugenicity:
   i) *In-vitro* metaphase analysis or
   ii) *In-vitro* micronucleus test*
3. Mammalian cell mutation assay (currently, preferred choice is the mouse lymphoma assay)**

(Test 3 is not required for those substances where there will be little or no human exposure.*)**

Footnotes:
* If there are indications of aneugenicity in the metaphase analysis (eg hyperdiploidy, polyploidy) or positive results in the micronucleus test there is a need to confirm whether the compound is an aneugen by use of appropriate staining procedures.
**A test other than the MLA may be used provided that it has equivalent biological relevance and equivalent statistical power.
***General guidance only is given. Decisions about whether, for example exposure to a specific substance is likely to be high, or moderate and sustained, would normally be taken by the Regulatory Authority on a case-by-case basis taking account of other relevant data.

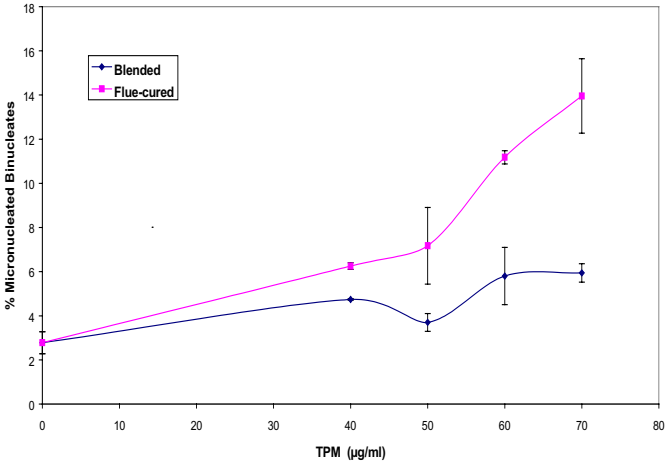
The *in vitro* micronucleus assay is based upon the principle of fragments, as a result of chromosome damage, or whole chromosomes that become detached from the mitotic spindle, are not left in the cell cytoplasm, but incorporated into micronuclei which can then be counted and dose response curves produced. As a technical aid in the assay, cytochalasin B is included which prevents cytokinesis i.e. division of the cell cytoplasm between the daughter nuclei following nuclear division. The micronuclei are then scored in binucleate cells, which confirm that cell division has taken place. The percentage of binucleate cells in the test population is also used as a marker of cytotoxicity in this assay.

Figure 10. Illustration of the Processes Leading to the Formation of Micronuclei



The effect of smoke condensates from a blended cigarette (Burley + flue-cured blend) and a flue-cured cigarette are illustrated in Figures 11 & 12. In contrast to the data from the Ames test, smoke condensates are positive in both the presence and absence of an S9 metabolic activating fraction. In the Ames test, tobacco smoke condensates are mutagenic only in the presence of the S9 metabolic activating fraction. Again, in contrast to the Ames TA98+S9 system, the condensate from the flue-cured condensate is more active in the *in vitro* micronucleus assay than that from the blended cigarette (cf. Figs. 11, 12, 13). Blended smoke condensate is more active than flue cured condensate in the Ames test.

Figure 11. Micronucleus Induction By Smoke Condensates In The Absence Of Metabolic Activation (-S9)



TPM = Total particulate matter

Figure 12. Micronucleus Induction by Smoke Condensates in the Presence of Metabolic Activation (S9).
TPM = Total particulate matter

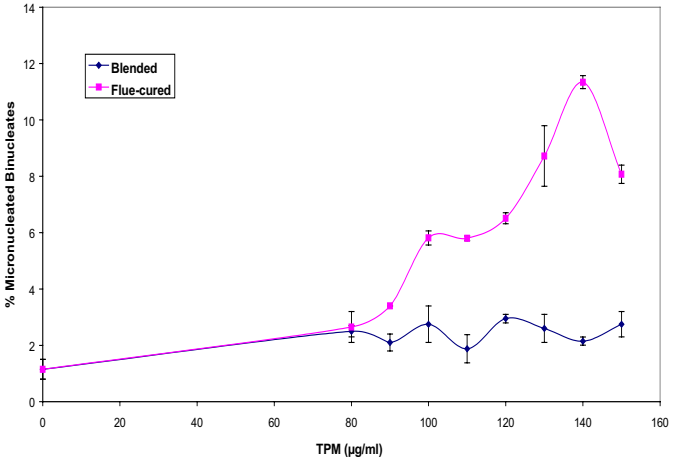
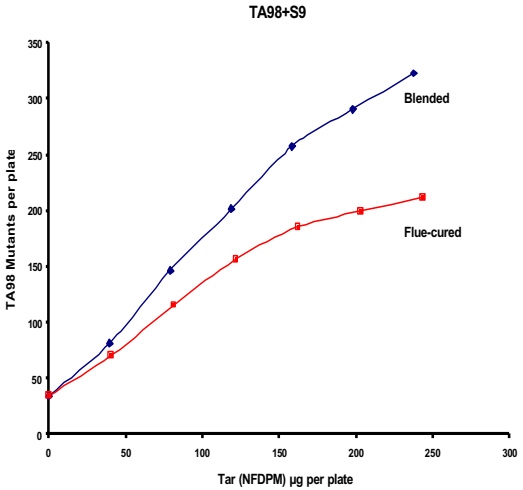


Figure 13. Ames TA98+S9 Activity of Smoke Condensates From Blended and Flue Cured Cigarettes.



These data would indicate that the mutagenic compounds in smoke condensates active in the two test systems, Ames versus chromosome damage in the form on micronuclei are not the same. Data are currently not available in the mammalian cell systems, to indicate which mutagenic agents in smoke condensate are responsible for the genotoxic activity.

To be able to differentiate between compounds that are clastogenic or induce numerical changes in chromosomes (aneugens) there is a need to demonstrate the presence of whole chromosomes (aneugens) versus chromosome fragments (clastogens) in the induced micronuclei. This is achieved by demonstrating the presence of the centomeric / kinetochore region of the chromosome, i.e., the point of the chromosome by which they

attach to the mitotic spindle during cell division, and which are present in whole chromosomes, but not fragments (24). Fluorescent stains linked to antibodies against the kinetochore consequently allow discrimination of the micronuclei with or without kinetochores (24 & 25).

Table 7. Kinetochore (KC) Positive (+ve) and Negative (-ve) Micronuclei in V79 Cells Treated with Smoke Condensate (25).

Dose µg ml^{-1}	% Micronucleated cells	% KC +ve	% KC –ve
Mitomycin C 0.08 (20 hr -S9)	11.84	22.4	77.6
Vincristine sulfate 0.08 (4hr –S9)	9.98	93.3	6.7
Tobacco smoke condensate (+S9)			
0	2.9	59.8	40.2
5	5.1	79.4	20.6
10	5.9	84.3	15.7
20	7.5	70.1	29.9
40	8.4	69.4	30.6

Table 7 demonstrates the effect of a known aneugen Vincristine and a clastogen, Mitomycin C. Tobacco smoke condensate using a protocol in the presence of S9 resulted in a dose related increase in the percentage of kinetochore positive micronuclei. Tobacco smoke condensate was consequently assessed as inducing both clastogenic and aneugenic effects (25).

4. Effects of Whole Smoke

The work described above has been carried out on tobacco smoke condensate added to the cell culture medium, which is in conformance with the majority of genetic toxicology experimentation. Tobacco smoke is

wt.) and experimentation is available examining the genetic toxicology of both whole smoke (particulate + vapor) and vapor phase alone (30).

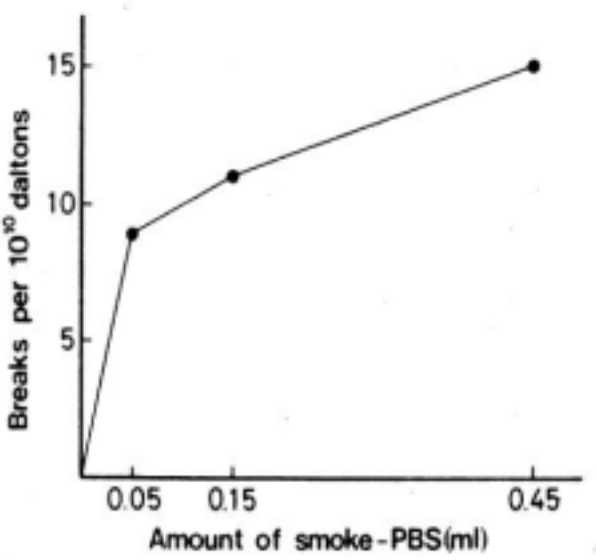
In the late 1970s, work by SEITA produced data on the gas phase of cigarette smoke. Smoke filtered through a Cambridge filter pad was bubbled through a saline solution and the solution then applied to lymphocytes in culture and sister chromatid exchanges measured (26). A dose response was noted with increasing number of cigarettes smoked into the liquid smoke trap and there seemed to be little difference in the effect if the lymphocytes were from smokers or nonsmokers (Table 8).

Table 8. Effect of a Vapor Phase Solution on Sister Chromatid Exchanges in Lymphocytes *in Vitro* (26).

	Treatments			
Lymphocyte source	**Control**	**0.06 Cig.**	**0.125 Cig.**	**0.25 Cig.**
	Exchanges lymphocyte^{-1}			
Smokers	5.96	8.46	12.81	20.05
Non Smokers	4.84	8.4	12.29	23.78

More recently, the group of Nakayama in Japan studied the formation of DNA single strand breaks in an A549 cell line exposed to a smoke solution produced by bubbling smoke through phosphate buffered saline (27). Whilst the efficiency of the trapping system was described as variable, using one preparation a dose response could be obtained and they also began to investigate potential chemical species responsible for the activity (Fig. 14).

Figure 14. Effect on DNA Single Strand Breaks of Increasing the Concentration of Smoke Solution in the Cell Culture Medium (27).



Adding either the enzymes catalase to inactivate the hydrogen peroxide found in the smoke solution (0.05 µg ml^{-1}) or super oxide disputes (SOD) to inactivate 'active oxygen' species inhibited the induction of single strand DNA breaks by the smoke solution by 84% and 36% respectively (Table 9).

Table 9. Effect of a Smoke Solution on Single Strand DNA breaks in A549 cells (27)

Treatment	SSBs / 10^{10} Daltons	% SSBs
Smoke solution	55.1	100
Smoke + SOD	35.4	64
Smoke solution	21.1	100
Smoke + catalase	3.4	16
Smoke + inactivated catalase	22.1	104

SOD - superoxide dismutase

In a subsequent paper by this group, they bubbled air through the smoke solution (degassed solution) and passed the gas through a second aqueous trap (gas solution) (28).

The degassed smoke solution lost its ability to induce DNA strand breaks, but measurements confirmed that the solution still contained the same amount of hydrogen peroxide and oxygen radical anions (Table 10). Furthermore, the gas solution was also inactive, but regained activity when hydrogen peroxide was added or recombined with the degassed smoke solution (Table 10). Further work on this effect does not appear to have been reported; though the conclusions indicate something more than hydrogen peroxide was involved in the DNA strand breakage activity of the smoke solutions.

Table 10. Effects of Degassing Smoke Solutions

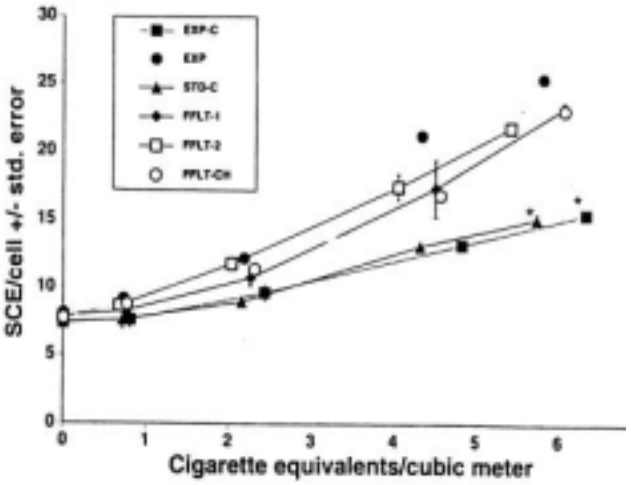
	SSBs / 10^{10} Daltons	**H_2O_2 µM**	**$O_2^{\cdot}$ OD_{560} #**
Smoke solution	7.7	8.5	0.069
Degassed solution	0	8.4	0.062
H_2O_2 (15µm)	0	-	-
Gas solution	0	-	-
Gas solution + H_2O_2	3.1	-	-
Gas solution + degassed solution	18.4	-	-

Absorbance after incubation with nitro blue tetrazoleum

More recent work on whole smoke systems have involved passing smoke over cell cultures in 75 cm^3 flasks, containing culture medium, but rocked backwards and forwards on a heated platform to expose the cells to the smoke (29). Induction of chromosome exchanges in the form of sister chromatid exchanges (SCE) was reported (Fig. 15). However, unlike the

Ames test, no differences were reported between smoke from cigarettes made up of blends with 'high' and 'low' nitrogen tobaccos.

Figure 15. The Effect on Sister Chromatid Exchange Following Exposure of CHO Cells in Culture to Whole Smoke (29).



Exp-C = Low nitrogen blend + new carbon filter

Exp = Low nitrogen blend + cellulose acetate filter

STD-C = Commercial blend + new carbon filter

FFLT-1 = Full flavor low tar

FFLT-2 = Full flavor low tar

FFLT-CH = Full flavor low tar + Charcoal filter

Work examining the induction of micronuclei is also available for a system

whole smoke or gas phase (31). Cells are cultured on 3µm pore Transwell membranes held in a smoke exposure chamber so that growth medium can be supplied to the underside of the membrane and smoke passed over the upper side on which the cells grow (Fig. 16). Smoke exposure of 24 sec per puff per minute for 3 hours can be carried out and an induction of micronuclei measured (Table 11).

Figure 16. Cross Section Through Smoke Exposure Chamber (31).

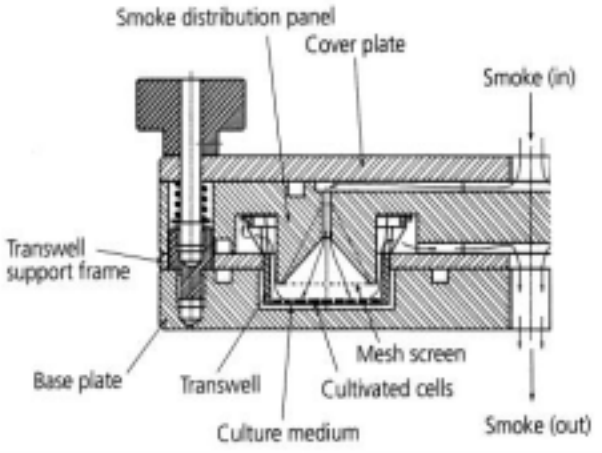


Table 11. Effects of Exposing V79 cells in Culture to Whole Smoke (31).

Particulate concentration mg m^{-3}	% Binucleate cells	% Micronucleated binucleate cells
Air (48)	77.6	1.87
133 (6)	50.0	3.80*
185 (6)	43.4	2.20
224 (6)	32.3	3.15*
457 (6)	25.2	4.12*
MMC (54)	48.8	8.89

* Air vs. smoke exposed $p<0.05$
( ) Number of replicate cultures

If a Cambridge filter pad is placed between the cigarette and the exposure block so that cells are exposed to vapor phase only, the data indicated that the mixtures were equally cytotoxic as demonstrated by a reduction in the percentage of binucleate cells in the test versus control population. The micronucleus frequency in this configuration was 76.5% of the value with whole smoke (Table 12). It should be stressed that these data are specific to this apparatus, where particulates are known to be deposited on the tubing of the system (~36%) and may reflect a whole smoke depleted in particulate phase. It nevertheless does demonstrate genotoxic activity of smoke vapor phase.

Table 12. The Effect of Whole Smoke versus Vapor phase (31)

Exposure	% Binucleate cells	% Micronucleated binucleate cells
Air (16)	90.4	1.28
Whole smoke 224 mg m^{-3} (15)	51.6	3.95*
Vapor phase (9)	55.9	3.02*#

( ) Number of replicate cultures
* $p<0.001$ vs. Air $p<0.001$ vs. Whole smoke

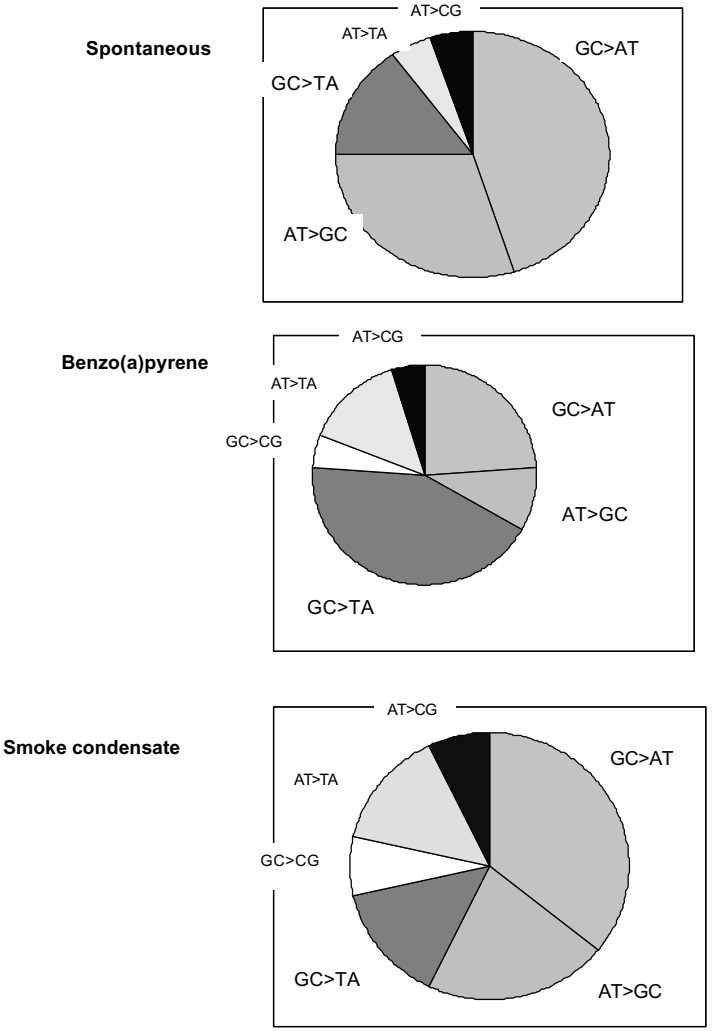
5. Mutagenic Spectra

With advances in molecular biology, it is now becoming possible to investigate the relationship between chemical exposure and the types of genetic lesions, and data is increasingly becoming available for many agents highlighting the specificity of mutagen interactions for target genes (33, 34).

Specifically in the *in vitro* field, metabolically competent cells lines (MCL-5) are available and have been used to investigate mutagen specificity using the hypoxanthine phosphoribosyltransferase (HPRT) gene (35). The HPRT enzyme usually converts hypoxanthine, guanine, and xanthine into their respective nucleotides. If cells are cultured in 8-azaguanine or 6-thioguanine, the HPRT enzyme will convert these to toxic nucleotides and the cells die. In cells that have been treated with mutagens and the HPRT locus mutated, the mutated cells are then deficient in the enzyme and consequently do not form the toxic nucleotides, will survive and can be isolated for further analysis.

The mutagenic spectra from untreated MCL-5 cells, smoke condensate treated cells and cells treated with benzo(a)pyrene are illustrated in Figure 17. In the benzo(a)pyrene treated cells the proportion of Guanidine to Thymidine transversions characteristic of polycyclic aromatic hydrocarbon treatment are increased (36). In contrast following the treatment by smoke condensate the distribution of lesions does not match that of the spontaneous or benzo(a)pyrene.

Fig 17. Base Changes in the HPRT Gene of MCL-5 Cells (35).



As in the experiments described above in the 1970s, work in the area of *in vitro* genetic toxicology continues to demonstrate a striving to understand the possible causative chemistry and the nature of lesions induced by tobacco smoke.

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ASSESSMENT OF TOBACCO SMOKE BY *IN VITRO* CELL TOXICITY ASSAYS

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Abstract

In vitro short-term tests provide rapid, economical means to evaluate the toxicity of tobacco smoke. *In vitro* genotoxicity assays are important to determine the effect of tobacco smoke on the genetic material within the cell. However, there are many nongenetic aspects of cellular physiology responsible for normal cell function that should be assessed during the toxicological evaluation of tobacco products. Nongenotoxic assessment of cytotoxicity after exposure to tobacco smoke is important since cell death and decreased cell viability may be indicators of increased cell proliferation, irritation, and inflammation *in vivo*. The neutral red (NR) cytotoxicity assay is a sensitive method that has demonstrated responsiveness to both particulate and vapor phases of tobacco smoke. The NR assay has been useful in determining the relative cytotoxicity of tobacco smoke from a variety of cigarettes as well as the reduction of cytotoxicity of tobacco smoke from potentially reduced exposure cigarettes (PREPS). Other nongenotoxic assays to assess tobacco smoke may be employed to examine more specific physiological mechanisms of the cell. Examples include assays that determine the ability of tobacco smoke to generate intracellular reactive oxygen species, disrupt intercellular communication, or destroy the integrity of cellular membranes. *In vitro* assays that measure cellular toxicity complement genotoxicity assays in providing a more complete *in vitro* toxicity assessment of tobacco smoke that is useful in product stewardship and the development of PREPS.

Introduction

Cigarette design has evolved over the last several decades. 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 the smoke yielded from the cigarette. RJRT continues to develop and evaluate novel technologies. RJRT has developed a tiered testing strategy to evaluate the potential for new ingredients, tobacco processes, and technological developments to increase or reduce the biological activity of cigarette smoke. This tiered testing strategy was created to provide a conceptual framework based on level of concern to facilitate the design of toxicological evaluation programs. Within the context of this framework, decisions regarding the design of a toxicological evaluation program are determined by considering two factors – the level of human exposure and the potential for toxicity. Combined, these factors, in conjunction with scientific judgment, provide a means to assess the appropriate level of concern. In brief, the level of concern is a relative measure of the extent to which a product modification may present a potential risk or a potential to present a reduced risk.

The level of human exposure reflects the level (frequency and magnitude) of expected exposure. The potential for toxicity is determined based upon available information (structure activity relationships, *in vitro*, *in vivo*). All data, including positive and negative findings, are considered to derive a weight-of-the-evidence conclusion.

Based upon the level of concern, an appropriate toxicological evaluation program is designed. For example, a modification determined to represent a low level of concern (such as a subtle change in the formulation of a cigarette paper) may require no further evaluation. By contrast, a modification determined to represent a greater level of concern (such as the

introduction of novel technology exemplified by ECLIPSE), as well as a large potential to reduce cigarette smoke toxicity, may require more extensive evaluation. Such an evaluation would include an examination of a number of smoke constituents (which have been identified by the Surgeon General and others to have the potential to contribute to the risks associated with smoking) as well as both *in vitro* and *in vivo* toxicology. When these tests are used to compare a product modification against current cigarettes, the results can be used in a weight-of-the-evidence approach to measure progress toward reducing the toxicity of cigarettes. This comparative approach requires generating data on either a reference cigarette such as the University of Kentucky reference cigarettes or generating data on a reasonable sampling of the existing cigarette market as described in publications from our laboratory (Steele *et al*., 1995; Putnam *et al*., 2001). It is important to emphasize that requiring all chemical or biological tests to be run on all brand styles in the market would be a huge waste of resources since extensive testing has shown that the composition of smoke and the resulting toxicity of current cigarettes are generally similar.

A comparative approach for measuring progress with cigarettes that have the potential to reduce risk against cigarettes currently on the market has been referred to as *the Rule of First Approximation*. This approach would not require a series of clinical studies (Phase I through Phase IV) similar to the testing required by the U.S. FDA to make therapeutic drug claims. This more pragmatic approach would rely on the weight-of-the-evidence from chemistry and toxicology compared to a suitable benchmark of marketed cigarettes to justify short-term clinical tests, which rely on surrogate markers. If progress can be demonstrated using this comparative approach, then appropriate claims about the potential for risk reduction would be permitted. This

approach would permit a more rapid introduction of modified cigarettes into the market and realization of the benefit of reduced risk products.

Following is a review of some of the non-genotoxic *in vitro* tests that have been used to evaluate cigarette smoke and cigarette smoke condensate. These analytical tools form a good basis for developing the type of tiered toxicological evaluation that R.J. Reynolds recommends.

Short-term In Vitro Assays

Short-term *in vitro* assays are ideally suited to the tiered testing strategy for measuring progress towards the reduction of cigarette smoke toxicity. They are widely accepted by regulatory agencies around the world, are fairly inexpensive, and rapid to conduct. There is an excellent mechanistic basis for believing that the endpoints measured in these tests are causally related to the risk of developing chronic disease. Therefore, short-term tests are amenable to studying a multitude of cigarette products and/or cigarette components. Smoke from new cigarette prototypes, cigarettes from the U.S. market, or Kentucky reference cigarettes may easily be studied. Whole cigarette smoke as well as condensate or other sub-fractions of the whole smoke are easily evaluated for toxicity in short-term tests. Additionally, one may examine the toxicity of sidestream smoke, mainstream smoke, or environmental tobacco smoke in these assays. These assays are also extremely useful for assessing the toxicity of isolated components in cigarette smoke, such as additives and flavors, as well as studying how the complex cigarette smoke mixture may affect the toxicity of isolated components or chemicals found in tobacco smoke.

Short-term tests can broadly be viewed as measuring two major endpoints: cytotoxicity and genotoxicity. Cytotoxicity is regarded as an important step in several chronic disease processes associated with smoking, including carcinogenesis and emphysema. These tests are

also very helpful for determining the appropriate exposure concentration for *in vitro* genotoxicity assays. Importantly the use of *in vitro* cytotoxicity assays minimizes the use of animals for toxicity assessment. The major endpoints evaluated in *in vitro* cytotoxicity assays in our laboratory and others include the effect of a test agent on cell viability and the effect of a test agent on cellular growth rates. Decreases in viability and/or growth rates are widely interpreted as evidence of cytotoxicity. Genetic toxicology, on the other hand, looks specifically at the effects of the chemical on the DNA molecule. The purpose of genetic toxicology studies is to characterize and define the potential of test agents to adversely affect the structure or function of DNA molecules. Genotoxicity has been mechanistically linked with carcinogenesis, both in experimental animals and in humans. Also, DNA damage in germ cells may produce heritable defects in offspring and DNA damage has been associated with the development of arteriosclerosis.

Tests to Assess Cytotoxicity

Cytotoxicity examines the toxic effects of chemical or physical agents on cellular structural or functional components. Biological endpoints for cytotoxicity may examine general mechanisms common to all cells (DNA synthesis, mitochondrial function, etc.) or mechanisms specific to a particular cell type (hormone secretion, metabolizing enzymes, neurotransmitter release, etc.) A list of general cytotoxicity biological endpoints can be found in Balls and Clothier, 1992.

Consideration of Cytotoxicity Assays for Industry and Government Toxicity Assessments

The use of cytotoxicity assays, as part of the toxicological assessment of a chemical or chemical mixture, is currently a very active area of consideration. Several large research groups have investigated the role of cytotoxicity assays in toxicology and have made recommendations

to industrial and government bodies. In the United States, the Center for Alternatives to Animal Testing (CAAT) at Johns Hopkins University has recommended several cytotoxicity tests for the safety testing of consumer products, such as cosmetics, detergents, and other personal hygiene products (Balls *et al*., 1990). A large multi-national effort entitled MEIC (multi-center evaluation of *in vitro* cytotoxicity) have examined a large number of cytotoxicity endpoints in a variety of cell types using a standardized list of 50 diverse chemicals with varying mechanisms of action. The MEIC studies have indicated a very good correlation with cytotoxicity and acute lethal potency and irritancy potential (Ekwall *et al*., 1994; Clothier *et al*., 1989; Ekwall *et al*., 1989). Another large research program, FRAME (Fund for the Replacement of Animals for Medical Experimentation), has identified several cytotoxicity assays including the neutral red test for the toxicological assessment of chemicals and potential pharmaceuticals (Faotrel *et al*., 1991; Knox *et al*., 1986; Rowan and Stratman, 1980).

Cytotoxicity assays are now being considered to replace the Draize eye test, and OECD Guideline No. 405 for acute eye irritation/corrosion has been modified for this accommodation. With respect to cosmetics, Directive 76/768/EEC, Article 4, has prohibited the marketing of products that have been tested on animals as of January 1998. This directive has required *in vitro* tests including cytotoxicity assays. Considerable effort has been made to validate the use of cytotoxcity assays for toxicity assessments. A summary of scientific criteria for the validation of in vitro toxicity testing has been compiled by CAAT, FRAME, and OECD (Frazier, 1992; FRAME, 1991, Balls *et al*., 1990; Organization for Economic Cooperation and Development, 1990).

Cytotoxicity Assessments of Cigarette Smoke and Cigarette Smoke Components

A number of our studies have been conducted in order to optimize assays conditions for the cytotoxicity assessment of cigarette smoke and cigarette smoke components. The neutral red uptake assay has been chosen from a number of cytotoxicity assays as being the most sensitive assay for assessing the cytotoxic potential of cigarette smoke condensates (Putnam *et al*., 1998; Bombick and Doolittle, 1992a; Putnam *et al*., in press). The neutral red assay has been optimized and used for cytotoxicity assessments comparing cigarette smoke condensates from cigarettes differing in "tar" amounts or different tobacco types (Bombick *et al*., 1998a; Putnam *et al*., 1997). The cytotoxicity of specific chemicals reported in cigarette smoke have been determined in the neutral red assay (Bombick and Doolittle, 1995) and recently, several phenolic and dihydroxybenzenes has been shown to have minimal contribution to the cytotoxicity of the entire cigarette smoke condensate mixture (D. Bombick *et al*., 1999).

The neutral red assay also has been used to validate an *in vitro* exposure system that permits exposure of cell cultures to the entire cigarette smoke comprised of vapor and particulate fractions (Bombick *et al*., 1997a; Bombick *et al*., 1991). The cytotoxicity of mainstream and sidestream smoke from various reference cigarettes was compared using this *in vitro* exposure system. In addition, this exposure system was used to expose cell cultures to a high level of environmental tobacco smoke with the result of no cytotoxicity observed in the exposed cell cultures (Bombick *et al*., 1991). The cytotoxic potential of cigarette smoke assessed from two recently developed cigarettes exhibited reduced smoke chemistries. A cigarette with a novel carbon filter exhibiting a significant reduction in vapor phase irritants (e.g., formaldehyde, acetaldehyde, acrolein, isoprene, etc.) also demonstrated a significant reduction in cytotoxicity of the whole smoke when compared to the smoke from a similar cigarette without the carbon filter

(Bombick *et al*., 1997b). Another cigarette that primarily heats tobacco exhibited significant reductions of chemicals when compared to tobacco burning cigarettes (Borgerding *et al*., 1998). The smoke condensate from the cigarette that heats tobacco (Eclipse) exhibited no cytotoxicity (Bombick, D. W. *et al*., 1998b; Bombick *et al*., 1996; Bombick *et al*., 1995). The whole smoke from Eclipse had similar cytotoxicity to an ultra-low "tar" reference cigarette and significantly reduced cytotoxicity compared to a light "tar" reference cigarette.

Significance of Cytotoxicity to *In Vivo* Situations

The significance of cytotoxicity to *in vivo* situations can be separated into four broad areas indicated as follows:

- Irritation and inflammation
- Cell proliferation and hyperplasia
- Oxidative stress and damage
- Decreased organ function

All these processes are dependent upon a cytotoxic injury which if chronically maintained can lead to a pathological condition. These processes may also initiate compensatory mechanisms that will lead to recovery or in some situations increase susceptibility of the animal or human to disease. The correlation of cytotoxicity with irritation and inflammation can be observed in several organ systems including the skin (Roguet, 1997; Roguet *et al*., 1994; Gueniche and Ponec, 1993; Cohen *et al*., 1991), eye (Spielmann, 1997; Balls *et al*., 1995; Osborne *et al*., 1995); the cardiovascular system (Ohno *et al*., 1994; Rossi *et al*., 1994; Roberts, 1995; Schaefer *et al*., 1995); and the pulmonary system (Li, 1986; Saladino *et al*., 1985; Li *et al*., 1983). Cell injury can induce cell proliferation and tissue hyperplasia as responses to inflammation (Stadnyk, 1994; Albelda *et al*., 1993; Argyris, 1987; Luger *et al*., 1985), wound healing (Border and Noble,

1994; Gailit and Clark, 1994; Stadnyk, 1994), and liver regeneration (Mehendale *et al*., 1994; Mehendale, 1991). Cytotoxicity and cellular injury that result in oxidative damage and the release of reactive oxygen species to other tissues can induce inflammatory (Varani and Ward, 1997; Varani and Ward, 1994; Halliwell, 1992) and hyperplastic responses (Butterworth and Goldsworthy, 1991; Amstad *et al*., 1990; Schulter-Herman *et al*., 1990). The necessity for tissues to reduce the oxidative stress can decrease normal physiological processes including metabolism (Samani *et al*., 1997). The release of reactive oxygen species may also lead to direct genetic damage and increased potential for mutations (Guyton and Kensler, 1993; Breimer, 1990; Floyd, 1990). In severe situations, cytotoxicity can destroy an organ system. Examples include ethanol toxicity and liver cirrhosis (Colton and Zakhari, 1997; Kurose *et al*., 1996; Bondy, 1992), chloroform toxicity and liver necrosis (Plaa, 1997; Cheeseman *et al*., 1985; Trivedia and Mowat, 1983; Klaassen and Plaa, 1969), and paraquat toxicity and decreased lung function (Krall *et al*., 1988; Bus and Gibson, 1984).

Involvement of Cytotoxicity in Three *in Vivo* Models

There are several *in vivo* models that illustrate the importance of cytotoxicity and cellular injury to a pathological process. Several of these models are described below:

Mouse Skin Painting Model

The mouse skin-painting assay examines the potential of a chemical or chemical mixture to induce skin tumors in the mouse. This assay has been used frequently as an animal test to assess the carcinogenic potential of chemicals (Boutwell, 1987; Slaga, 1986; Boutwell *et al*., 1981). Some of the chemicals that are positive in this assay are cytotoxic and have been identified as tumor "promoters." The cellular injury induced by these chemicals results in the proliferation of cell populations that may have a mutated phenotype and ultimately produce a

malignant tumor. The classic tumor-promoting agent, phorbol 12-myristate 13-acetate is cytotoxic and causes the generation of reactive oxygen species, skin inflammation, and a skin hyperplasia (Kensler *et al*., 1989; Cerutti, 1985), all of which are mechanisms previously described as possible outcomes from cytotoxicity. Other cytotoxic chemicals including benzoyl peroxide, mezerein, and canthardin cause similar effects in skin and are skin tumor promoters in the mouse skin-painting assay (DiGiovanni *et al*., 1987; O'Connell *et al*., 1987; Slaga *et al*., 1983; Slaga *et al*., 1981).

Atherosclerosis Model

An atherosclerosis model illustrates the involvement of cellular injury in the potential development of atherosclerotic plaques. Endothelial cells within the pulmonary system are directly susceptible to reactive oxygen species present in the lung (Cross, 1987), which could lead to compromised pulmonary vasculature. Also, oxidized low-density lipoprotein formed by macrophages is reported to be cytotoxic to endothelial cells (Mehra *et al*., 1995; Dzau, 1994). The initial damage to arterial endothelial cells by a cytotoxic mechanism may be the initiation of the sclerotic process involving formation of arterial fatty streaks, deposition of foam cells, and the ultimate manifestation of an atherosclerotic plaque (Dzau, 1994; O'Brien and Chait, 1994). In addition, thrombogenic events can incur cellular damage to endothelial cells to result in a similar arterial lesion (Dzau, 1994; Nachman and Silverstein, 1993; Rubanyi, 1993).

Pulmonary Inflammation Model

A pulmonary inflammation model illustrates the interactions of several cell types and the pathological ramifications if one or more of these cell types are injured. Alveolar macrophages are an important pulmonary cell type with a key role in mediating the inflammatory response and maintaining the integrity of the lung parencyma (Fels and Cohn, 1986). Macrophages activated

or killed by chemicals (Oosting *et al*., 1990; Graham and Gardner, 1985; Sone *et al*., 1983; Gardner and Graham, 1976) or particles (Dixit *et al*., 1990; Bowden, 1987; Allison, 1974) can secrete or release proteolytic enzymes and reactive oxygen species, which can injure surrounding pulmonary cells and result in an inflammatory response (Nathan *et al*., 1980; Harris *et al*., 1970). Similarly, damage to Type I and Type II alveolar epithelial cells may result in the release of cytokines responsible for the recruitment of macrophages (Driscoll *et al*., 1990; Sibille and Reynolds, 1990; Wolff *et al*., 1990; Nathan *et al*., 1980). Damage to Type I and Type II cells may also cause genetic changes or an abnormal proliferative response of these cells within the alveoli (Driscoll *et al*., 1994; Bond *et al*., 1990; Witschi, 1976). Several of the pulmonary cell types (e.g., Clara cells, alveolar macrophages, etc.) have significant metabolizing capability that can activate chemicals to a more cytotoxic form (Chichester *et al*., 1991; Forkert *et al*., 1989; Rietjens *et al*., 1988; Domin *et al*., 1986; Devereux, 1984; Jones *et al*., 1982; Devereux *et al*., 1981). Chemicals that injure these various pulmonary cell types to induce a pulmonary inflammation may also be associated with chronic disease processes such as emphysema and cancer (Phan and Thrall, 1995; Toyokuni *et al*., 1995; Yeadon, 1995; Kamp *et al*., 1992; Kovacs, 1991; Shaw *et al*., 1991; Haslett and Henson, 1988; Fedullo and Ettensohn, 1985; Crystal *et al*., 1984; Devereux, 1984; Swenberg *et al*., 1983; Jones *et al*., 1982; Brody, 1981; Hunninghake *et al*., 1979).

Other Nongenotoxic Endpoints

Cytotoxicity assays (and the appropriate genotoxicity assays) are often adequate first-tier short-term tests to assess tobacco smoke for product stewardship or the development of potentially reduced risk cigarettes. There are additional short-term tests that can be used to investigate possible mechanisms of toxicity. These mechanisms may be involved in cell death,

cell proliferation, or alterations in the cells genetic material. Changes in plasma membrane characteristics can be a mechanism involved in cytotoxicity and have been used as an index of *in vitro* toxicity (Bombick and Doolittle, 1990). Changes in mitochondrial membrane potential also can be used as an indicator of cytotoxicity (Rahn *et al*., 1991). Gap junctional intercellular communication (GJIC) is a process that normal cells use to maintain homeostasis within tissues and organs. Assessment of GJIC has been used to compare exposure of several different cell types to a K1R4F cigarette smoke condensate and a smoke condensate from Eclipse cigarettes or a variety of other chemicals classified as tumor promoters (McKarns and Doolittle, 1991; McKarns *et al*., 1991; McKarns *et al*., 2000). Finally, additional *in vitro* assays can examine the oxidative stress incurred by compounds on cells. Current fluorescent techniques can be used to detect the presence of reactive oxygen species within cells after exposure to oxidants (Bombick and Doolittle, 1992b).

Summary

In summary, these short-term tests complement existing genotoxicity tests and are easily adapted to evaluating the potential of cigarette smoke and/or its components to adversely affect the structure or function of biologically important molecules. Additionally, these tests have demonstrated their usefulness in assessing reduction in the toxicity of cigarette smoke due to product modifications.

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THE TOXICOLOGICAL ASSESSMENT OF TOBACCO PRODUCTS: *IN VIVO* APPROACHES

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1. INTRODUCTION

The great majority of toxicologists and prominent professional societies acknowledge that in the absence of human data, research with experimental animals is the most reliable means of detecting the toxic properties of chemical substances and for estimating risks to human health (SOT 1999). There is also a near-universal appreciation of the need for responsible treatment of research animals; research designed to reduce, refine, or replace animals with validated *in vitro* assays or even computer-aided structure/activity predictions of toxicity is currently funded at record levels. Still, there remains a broad consensus that *in vivo* studies of biologically active chemicals and complex mixtures will for the foreseeable future continue to serve an essential role in advancing our understanding of biological mechanisms and in predicting the occurrence of human toxicity and disease.

Attempts to replicate human smoking-associated diseases in experimental animals began soon after those associations were first made in the epidemiological literature. The present brief review will focus on several of the primary *in vivo* toxicology methods that account for much of the vast experimental smoking and health literature. No two toxicologists will agree completely in regard to how these admittedly imperfect tools may best be used, or their results interpreted, to address the complexities of tobacco smoke exposure and effects. Nevertheless, until better means to develop an understanding of the toxicity of cigarette smoke emerge, we shall see a

continuation of *in vivo* experiments involving the exposure of living animals to cigarette smoke by the inhalation route, to various fractions of the smoke aerosol by topical application, or to tobacco and smoke constituents by other routes of administration.

2. QUESTIONS ADDRESSED BY *IN VIVO* STUDIES OF TOBACCO AND TOBACCO SMOKE

The three primary reasons for the conduct of *in vivo* toxicology studies of tobacco smoke that were itemized and discussed in a 1974 NCI workshop (Gori 1974) remain valid today. These are: 1.) The study of the mechanism of action of whole smoke or smoke constituents, 2.) The study of the pathogenesis of specific diseases and the development of early endpoints predictive of disease outcomes, and 3.) The screening of less hazardous cigarette models by characterizing their quantitative responses in terms of predictive endpoints related to specific diseases.

Product Stewardship

Additionally, *in vivo* studies have more recently been used as elements of product stewardship programs to evaluate the potential of added cigarette flavor and humectant ingredients to affect the inherent toxicity of cigarette smoke (Gaworski et al. 1998; Gaworski et al. 1999; Carmines 2002; Vanscheeuwijck et al. 2002; Heck et al. in press 2002; Ayres, Pence, and Mosberg 2001). While animal toxicology studies are a prominent component of the regulatory approval process for analogous substances such as direct food additives, these studies of tobacco product ingredients have frequently been performed in the absence of any regulatory requirements or guidelines. Emerging regulatory oversight of ingredients added to tobacco in the European Union and elsewhere indicates a continuing and substantial need for such *in vivo* studies in the future. Governmental bodies will have to weigh their perceived need for greater

scrutiny of tobacco ingredients against the demands of "animal rights" activism that has previously constrained scientists' ability to perform many tobacco-related studies in some jurisdictions.

Product Design

The last few years have also seen the presentation of *in vivo* toxicology data in documentation of the efficacy of novel cigarette designs in reducing the delivery of toxic smoke constituents (RJRT 1988; Wagner et al. 2000). These recent developments signal a timely renewal of the significant efforts of the National Cancer Institute's Smoking and Health Program of 1968-1979 (Gori 2000). This remarkable project included the participation of industry, academic and government scientists in an ambitious effort directed toward reducing the delivery of undesirable smoke constituents by technological means, with documentation of the effects of the modifications by smoke analysis as well as the best available *in vitro* and *in vivo* models of the day (Gori 1976; Gori 2000). Dr. Gori's retrospective (2000) on the Program, as well as the available technical reports of biological test results for some 150 cigarette construction and compositional variables, should be considered required reading for the next generation of scientists active in these areas. Regrettably, the abrupt discontinuation of the Program prevented the completion of many of the key *in vivo* assessments that had been planned for the project. However, the subsequent application of chronic *in vivo* smoke inhalation and mouse skin painting models in the evaluation of the tobacco substitute Cytrel exemplified a continuity of effort in the linkage of analytical measures of reduced delivery to *in vivo* biological endpoints in this first wave of reduced-exposure design concepts (Coggins, Lam, and Morgan 1982; Coggins et al. 1982).

The advisory opinion offered to the U.S. Food and Drug Administration (FDA) in the recent report of the Institute of Medicine Committee on Tobacco Harm Reduction provides more contemporary evidence that many of the concepts underlying the NCI program of the 1970s remain viable. The Report also confirms that *in vivo* toxicology testing will likely comprise an integral component of any future regulatory approval of claims for products designed to have reduced deliveries of one or more undesirable smoke constituents (Stratton et al. 2001). The Institute of Medicine convened this committee in late 1999 to address a request from the U.S. FDA for an advisory opinion on how that agency should regulate what it perceived to be implied medical claims for novel, reduced-exposure tobacco products that were beginning to appear in test markets in the United States (Stratton et al. 2001). The third of five general *Research Conclusions and Recommendations* developed by this Committee called for "*...the development of appropriate animal models and in vitro assays of the pathogenesis of tobacco-attributable diseases...*" to facilitate the meaningful laboratory evaluation of novel reduced-exposure/reduced-harm tobacco products. This call for the development of adequate animal models today, so many decades after the first *in vivo* studies of tobacco were begun, is testament to the reality of the remaining uncertainties regarding the pathogenesis of tobacco-associated diseases and, properly, the continuing reexamination of the relevance of available *in vivo* experimental methods to these processes.

3. CIGARETTE SMOKE INHALATION STUDIES

The findings of chronic mainstream cigarette smoke inhalation studies in rats, mice, hamsters, dogs, and non-human primates have recently been reviewed (Coggins 1998, 2001). The studies included in these reviews are in addition to dozens of other (generally early) studies that were of shorter duration, lacking in histopathological analyses, incompletely reported, or

that otherwise failed to meet inclusion criteria. These recent reviews of chronic cigarette smoke inhalation studies in five species showed that such studies have not been consistently and convincingly demonstrated to produce a significant lung tumor response, despite the strong epidemiological evidence supporting an expectation of such a response. Nevertheless, the unique nature of the exposures that accompany human smoking generally renders the more common oral and parenteral routes of test article administration much less appropriate, and so attempts to realistically model human tobacco smoke inhalation in experimental animal studies should and will continue.

Methodological Considerations

An accurate quantification of the delivered dose is of paramount importance in any *in vivo* toxicology study. Inhalation studies pose unique difficulties in this regard, since the internal dose resulting from an aerosol exposure is not simply the concentration of that aerosol presented to the animal, but also a function of the animal's respiratory rate (minute volume), the deposition efficiency of the inhaled test material, and the duration of the exposure. The use of *in vivo* smoke inhalation models to meaningfully study, understand, predict, and reduce the occurrence of human toxicity is usually most informative when a number of additional considerations are kept in mind:

Selection of exposure levels. Selection of an informative upper smoke exposure range to be tested in an animal study is recognized to be important in providing the investigator with a likelihood of producing interpretable data at the end of a costly and time-consuming animal study. The levels of exposure to a test article in most *in vivo* toxicology studies are typically substantially higher than those experienced by humans under the real-world exposure scenarios of interest in order to produce, within a relatively short time, a statistically measurable

occurrence of a biological response in a relatively small population of 10, 20, or perhaps 50 test animals of each sex. Published guidelines for maximum dose selection are available for the design of subchronic or chronic bioassays of pharmaceuticals or similar test articles in the preclinical stages of development (ICH 1994). However, the nature of the questions addressed in most *in vivo* testing of tobacco smoke are often very different from those that underlie most toxicology tests, so the selection of doses or smoke exposure levels is much more a matter of the principal investigator's judgment. Clearly, though, there is an upper limit to smoke exposure concentration that in most instances will be established by the accumulation of carbon monoxide as carboxyhemoglobin in the blood.

A study intended to compare the toxicity of the smoke of one cigarette to another might call for a maximally-tolerated, high-level smoke exposure to produce a measurable response in an endpoint of interest, such as the appearance of a low-level DNA or hemoglobin adduct. In other instances, an intermediate level of exposure might be indicated to ensure that an overwhelming incidence or excessive severity of respiratory tract histopathologic lesions does not obscure the detection of histopathologic changes of a more informative and quantifiable nature for the purpose of a cigarette-to-cigarette comparison. For example, a subchronic rodent cigarette smoke exposure to 1200 mg/m^3 smoke particulate material for 1-2 hours per day might be reasonably well tolerated in terms of gross measures of toxicity such as animal survival or body weight depression. However, a comparative assessment of a histopathological endpoint such as might be desirable in an evaluation of the potential effects of added flavoring ingredients, is probably more meaningfully performed at a more moderate exposure level of perhaps 350 mg smoke particulate material/m^3 (Gaworski et al. 1997; Gaworski et al. 1998).

Most contemporary subchronic cigarette smoke inhalation experiments are conducted in accordance with the general and applicable EPA and OECD guidelines for inhalation studies, with attention to the special considerations required for this unique test article. Typical exposure protocols, such as might be employed to compare responses between cigarettes, specify one or two daily one-hour smoke exposure sessions, five to seven days per week, for a period of 13 weeks (90 days). Various combinations of daily exposure duration, smoke concentration, and total study length have been evaluated over the years (Chen et al. 1989), and adjustment in any of these exposure variables may be indicated by the investigator's priorities in maximizing detection of a particular response, minimizing study completion time, or reducing overall study expense. A recent report (Kaegler et al. 2001) is interesting in this regard in that it suggests that a one to two hour daily smoke exposure of rats is similar, or possibly superior, in producing an array of characteristic responses to cigarette smoke when compared to a far more difficult and costly 6-hour daily exposure protocol.

A key element of any such study is the provision for the inclusion of several levels of exposure in order to provide a basis for the evaluation of a dose-response relationship for any observed responses. The design of studies intended to evaluate the potential toxicity of added cigarette ingredients introduce special complications in this regard. Since tobacco smoke itself comprises the vehicle for the delivery of volatilized ingredients or their pyrolysis products in a cigarette smoke inhalation study, the investigator must attempt to discriminate dose-related tobacco smoke effects from any potential effects of the tested ingredients of interest. In this instance, the exposure of several groups of animals to a single smoke concentration generated from matched control and test cigarettes containing various levels of the tested ingredients may be employed to evaluate any manifestations of toxicity attributable only to the presence of the

added ingredients (Carmines 2002). Further complications arise when tested ingredients are added to test cigarettes in quantities sufficient to displace a significant mass of the tobacco filler. Any specific effects attributable to the presence of the tested ingredients of interest must be discerned from those that may arise simply from the reduction in the quantity of tobacco burned in each puff consequent to the inclusion of exaggerated quantities of tested ingredients.

Smoke exposure mode. Animal smoke inhalation studies demand consideration of a number of factors peculiar to this test article and route of administration. These complexities render cigarette smoke inhalation studies difficult and expensive to perform well; and as a result, there are only a handful of laboratories in the world with the capability to conduct such work. Some of the relative advantages and disadvantages of two common modes of presentation of cigarette smoke to experimental animals, *whole-body exposure* and *nose-only exposure*, are briefly listed below.

Since cigarette smoke is a chemically and physically dynamic aerosol, a cigarette smoke exposure apparatus should be configured to deliver smoke with as little aging as possible. Studies of simulated environmental tobacco smoke (ETS), on the other hand, should ideally include some means to realistically dilute and age sidestream smoke or sidestream/mainstream mixtures to simulate the chemical and physical phenomena that occur in real-world ETS environments (Coggins et al. 1993).

Animals such as laboratory rats and mice exposed to cigarette smoke in a whole-body chamber may take in orally a considerable quantity of smoke particulate material deposited on the fur as a consequence of grooming behavior, both during the exposure and in the periods between smoke exposure sessions. This oral intake as well as direct dermal absorption may substantially compromise protocol-specified assessments of internal biomarkers of inhaled dose

and effect. Plasma nicotine and cotinine levels are disproportionately high, on the order of a factor of 3 (Chen et al. 1995), in chamber-exposed animals compared to those exposed by a nose-only procedure in which fresh smoke is introduced and exhaled smoke is extracted from the breathing zone of the animal. Other disadvantages of the whole-body exposure technique include physical and chemical changes in the aging smoke aerosol within the exposure chamber and the rebreathing of some portion of the residual smoke exhaled by the animal. Advantages of the whole-body exposure mode include lower labor and overall study costs, and reduced stresses on the unrestrained animals that in turn permits longer daily smoke exposure sessions, for a potentially increased cumulative delivered dose. However, any actual value of protracted daily exposures, such as the six hours per day suggested in OECD Guideline 413, has not proven to be readily demonstrable in cigarette smoke inhalation experiments comparing a six-hour continuous exposure to two daily 1-hour exposures at comparable weekly TPM doses (Kaegler et al. 2001).

Many contemporary investigators prefer to employ a nose-only mode of animal exposure to mainstream cigarette smoke, in which the animals are restrained in tubes as a continuous supply of fresh smoke is introduced only into the breathing zone of the animal. Smoke is continuously extracted at an appropriate rate to prevent rebreathing of exhaled smoke by the same animal or other animals exposed concurrently. The freshly generated smoke atmosphere produced by a contemporary automatic smoking machine/nose-only exposure apparatus is typically presented to the test animals within seconds of its generation and thus is arguably more realistic than the aerosol found within a whole-body chamber. Deposition of smoke particulate material on the pelt, with subsequent oral intake, is minimized in the nose-only exposure condition. This provides the investigator with the opportunity to more accurately quantify the inhaled dose through the use of particle-associated biomarkers such as blood nicotine and

cotinine. On the other hand, the animal restraining tubes required to achieve a nose-only exposure impose stress on the animals, which can contribute to losses in body weight and induce biological effects associated with the activation of the neural and endocrine components of the stress response. This disadvantage is addressed in the design of modern restraint devices to minimize thermal and physical stress effects. Any residual effects of restraint stress may in any event be largely controlled for with proper sham-exposed control animals that are restrained similarly but exposed only to air rather than to the experimental smoke atmosphere.

Selection of test species and strain. Dozens of purpose-bred strains of experimental animals are available to the contemporary *in vivo* toxicologist. The use of a particular animal strain that has been maintained for many generations offers the advantage of minimizing animal-to-animal variability by ensuring that all animals within a study are as genetically identical as possible.

Rats and mice offer the advantages of conveniently small size and relatively low animal and housing costs. Rats are usually preferred over mice for histopathological comparisons due to their somewhat greater response to cigarette smoke (Wehner 1989). This slightly larger species is also usually preferred for measurement of the effects of smoke on breathing patterns (Coggins et al. 1981), as well as for procedures involving the enumeration of free lung cells or evaluations of lung fluid biochemistry. The restricted lung volume of the mouse limits the quantity of lavage fluid that can be collected for analysis. Most contemporary comparative cigarette smoke inhalation studies employ one of a number of well-characterized strains of rats (Gaworski et al. 1997; Gaworski et al. 1998; Coggins 1998; Vanscheeuwijck et al. 2002; Heck et al. in press 2002) and less frequently mice or other non-rodent species (Coggins 2001).

A new inhalation model for cigarette smoke tumorigenesis? The Holy Grail for cigarette smoke inhalation toxicologists would unquestionably be a meaningful animal inhalation model for the development of lung tumors having a histological and etiological resemblance to the major human lung cancers. In light of the failure of the many previous attempts to produce a significant tumorigenic response in rodent cigarette smoke inhalation studies, it is of interest to note the very recent report of Witschi and colleagues at the University of California, Davis (Witschi et al. 2002).

The report of Witschi (2002) briefly reviews a series of small, whole-body inhalation studies of mainstream and/or sidestream smoke that reportedly yielded an elevated incidence and multiplicity of lung adenomas in the A/J mouse. These spontaneous or chemically enhanced tumors of the 'A' mouse strain may be scored visually at necropsy, as they occur close to the translucent outer surfaces of the mouse lung. While a full review of the model is a topic unto itself, it may be useful for the present discussion to point out that the key and essential element of the system appears to reside in a novel smoke exposure regime. The protocol specifies a cigarette smoke exposure period of about five months followed by a maintenance/recovery period of about four months without smoke exposure before tumor analyses are performed (Bogen and Witschi 2002). This is in contrast to many previous and unsuccessful attempts to produce lung tumors in rodent species, including the A/J mouse (Finch et al. 1996), in protocols that have employed conventional daily smoke exposure periods of up to the animal's lifetime with necropsy carried out immediately following the exposure period.

The strain 'A' mouse model had fallen out of favor some years ago after validation studies of its potential as a short-term *in vivo* screening method to detect carcinogens revealed a number of substantial shortcomings in terms of its lack of specificity and extreme variability, in addition

to the difficulties inherent in the use of a strain having a spontaneous lung tumor incidence approaching 100% in untreated control animals (Wagner 2001). Furthermore, the mechanistic basis for some of the previously reported findings from tobacco smoke exposures in this system remain to be fully understood. For example, the A/J mouse model indicates that the vapor phase of simulated environmental tobacco smoke is just as effective as the whole smoke in producing the tumorigenic response (Witschi et al. 1997). Nevertheless, Witschi's 2002 paper is intriguing in that it provides a very preliminary indication from Balb/c and SWR strains that the emergence of mouse lung adenomas subsequent to a subchronic tobacco smoke exposure, followed by several months of recovery, may occur in mice other than the 'A' strain. Substantial additional development, refinement, and validation of the smoke exposure/recovery protocol with larger studies, characterization of a dose-response relationship, and a better understanding of the mechanisms underlying the response will be required before its utility as an informative model of tobacco smoke inhalation can be determined.

Effects of Smoke Inhalation in Animal Models

Smoke deposition in the respiratory tract. The common laboratory rodent species are obligate nose-breathing animals having highly developed and complex olfactory structures in the upper respiratory tract. As might be expected, significantly more smoke particle deposition, smoke vapor phase chemical interaction, and histopathologic change occurs in these upper respiratory regions than is typical for human smoking patterns in which smoke is introduced directly through the pharynx and into the lower respiratory regions. It is for this reason that the smoke-associated histopathologic changes of greatest interest in comparative cigarette smoke inhalation studies in rats and mice frequently occur in the nasopharynx and larynx rather than in the lungs themselves.

One may classify chemical substances such as vapors and gases by the site of the manifestation of their local toxic effects in the respiratory tract, and these sites of action are in turn determined largely by the chemical and physical characteristics of the agents themselves. Upper respiratory irritants affecting the pharynx, nose, and associated olfactory regions are typically substances of high water solubility that are able to diffuse readily into the aqueous mucus layer lining the respiratory tract. Smoke constituents such as ammonia, formaldehyde, and acrolein are representative upper respiratory irritants and might be expected to contribute significantly to the manifestations of smoke toxicity that are observed in the upper respiratory tract of experimental animals. These irritants do have significant effects on respiration, which in turn can introduce complications in studies that include the comparison of two different cigarettes (Coggins et al. 1981).

Deep lung irritants include gases and vapors of lower water solubility and some compounds present in small smoke droplets that tend to be carried deep into the alveolar region of the lung that is the site of gaseous exchange. The site of deposition of inhaled particles is highly dependent upon the mass median aerodynamic diameter of the particulate aerosol, such that larger particles are generally less able to reach the deeper regions of the respiratory tract. However, substantial smoke particle deposition, in excess of that predicted by modeling based solely on particle aerodynamic diameter, occurs in the upper lung regions at the bifurcations of the bronchi and larger conducting airways in both humans and animals. The particulate droplets found in freshly generated cigarette smoke are largely of a submicron size range and thus are also carried readily into the deeper regions of the lung for additional deposition by diffusion and settling. The penetration of cigarette smoke particulate material into the deep lung areas of nose-breathing rodents might logically seem to be markedly less efficient than that resulting from

voluntary human smoking. However, experimental studies employing chemically- and radioactively-labeled smoke particles have documented the efficient experimental delivery and deposition of 60% to over 90% of retained smoke particle label below the level of the trachea of rodents (Binns et al. 1978; Kendrick et al. 1976; Chen et al. 1989; Chen et al. 1995).

Pathology. As might be anticipated, the tissues and organs of the respiratory tract are of predominant interest in the histopathological analyses conducted for the majority of tobacco smoke inhalation studies, although a complete protocol includes a comprehensive examination of all of the major organ systems, biochemical and hematological parameters, and body weight effects.

Rats exposed to moderate to near-maximum tolerated levels of cigarette smoke for a few weeks or for a lifetime show similar qualitative responses in terms of respiratory tract histopathology. The severity or extent of certain of the observed changes increases with smoke concentration and the duration of smoke exposure. Early changes include *hyperplasia* (an increase in cell number) of the various epithelial surfaces lining the upper and middle levels of the respiratory tract; this change is observed microscopically as a thickening of the lining of the surfaces of the nose, larynx, and trachea. Higher-level or longer-duration exposures result in *metaplasia*, or a change in cell type, including *squamous metaplasia*, whereby the cells assume a microscopic appearance resembling that of the skin. Cells in regions having *cilia*, the hair-like projections of epithelial cells that propel the overlying protective layer of mucus upward to clear particulate contaminants from the respiratory tissues, may experience a loss of ciliary function in response to cytotoxic smoke components such as carbonyls. These smoke-exposed ciliated cells may eventually lose their cilia altogether and be replaced by a nonciliated epithelium. *Goblet cells*, which produce mucus in these tissues, may become more numerous and exhibit an altered

appearance (*goblet cell hyperplasia/metaplasia*) as they produce larger amounts of the protective mucus layer in response to repeated high-level smoke exposures. Most of these changes have been described as adaptive responses to repeated exposures to the irritation of smoke rather than premalignant changes in light of the many demonstrations over the years that continuation of smoke exposure throughout the animals' lifespan does not result in a significant progression of these lesions to a cancerous state (Burger et al. 1989).

A small region of the larynx, found in cross sections where both the vocal cords and the sub-mucosal glands are visible, has been identified as the most sensitive and responsive region to histopathologic change in the rat (Sagartz et al. 1992). The typical incremental responses to smoke described above appear earliest and at the lowest levels of smoke exposure at this site, so this area should routinely receive particular attention in histopathologic evaluations.

The tissues of the lungs typically show a fairly limited spectrum of histopathologic changes consequent to cigarette smoke exposure in experimental animal studies. Accumulations of *macrophages*, cells that serve to engulf and clear deposited particulate matter from the lung, are a common finding. These cells often exhibit a brown or brown-gold color under microscopic examination, probably due to the presence of cigarette smoke particulate matter that has been phagocytosed or engulfed by the macrophage cells. In general, the changes described above are highly reversible, although the duration of the persistence of certain of the observations, such as alveolar accumulation of macrophages, has yet to be completely characterized.

4. SKIN PAINTING BIOASSAYS

The investigations of Roffo (Roffo 1937) and Sugiura (Sugiura 1940) were among the earliest demonstrations of a tumorigenic response by cigarette smoke preparations on the skin of experimental animals. These and subsequent studies in mice through the 1940s and 1950s were

briefly reviewed by Wynder and colleagues (Wynder, Graham, and Croninger 1953) in their report describing a mouse skin painting study of cigarette "tar" that had been performed by a protocol similar to one that had proven to be informative in work on another complex mixture, coal tar, and its fractions. While several laboratory animal species including rabbits, hamsters and rats have been evaluated for responsiveness to chemical carcinogens; the mouse has proven to be most sensitive to the multistage process of skin carcinogenesis and has been by far the most-used *in vivo* model.

Mouse skin painting bioassays of cigarette smoke condensate (CSC) were precisely that in a number of the early studies. Solutions of cigarette smoke solids were applied semi-quantitatively with a small artist's paintbrush to achieve delivery and disposition of the test article over a few square centimeters of the animal's shaved dorsal skin. Contemporary protocols typically employ a repeating pipette device to administer the test smoke condensate in acetone solution, followed by a deft stroke of the pipette tip wielded by an experienced technician to spread the dose evenly over the skin. Administered quantities of 10 to 50 mg CSC per application, two to five times per week, will typically result in the appearance of benign wart-like papillomas within a few months, with progression in tumor size, number, and histological character to produce a smaller number of malignant squamous cell carcinomas.

The mouse skin painting test system offers a number of advantages, including the production of a readily scored benign and/or malignant tumor endpoint within a reasonably short experimental duration (typically 6-12 months). Certain information on the mechanism of action of active substances or mixtures may be developed from protocols designed to characterize the activity of tumor initiators, tumor promoters, co-carcinogens, or complete carcinogens. Unique to this test system is the ability to quantify tumor number, size, and progression to malignancy on

an ongoing basis on the external skin of the living test animal rather than at a fixed time point in an internal organ accessible only at terminal necropsy. Historically, this model has provided the only experimental means to reliably produce tumors in statistically meaningful numbers with cigarette smoke preparations.

Epidermal neoplasia can be demonstrated in mouse dermal bioassays by either of two basic protocols, *i.e.*, *complete carcinogenesis* or *two-stage* (initiation & promotion) *carcinogenesis* (Boutwell 1964, 1974). The typical complete carcinogenesis protocol entails the repeated application of a test article such as tobacco smoke condensate onto the shaved dorsal skin of susceptible strains of mice. A typical two-stage carcinogenesis protocol specifies that a subthreshold dose of a known carcinogen {e.g., benzo(a)pyrene [B(a)P] or 7,12-dimethylbenz(a)anthracene (DMBA)} that is insufficient in itself to produce tumors is applied as an *initiator*; and followed by multiple applications of a *promoter.* Promoting agents are classically noncarcinogenic substances such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) that possesses an ability to induce epidermal hyperplasia (DiGiovanni 1994). Substitution of either the known initiator or the known promoter with a test article of interest, such as a smoke condensate preparation or fraction, provides an appraisal of the capacity of that test article to act as an initiator, a promoter, or both.

Subsequent work with the mouse skin tumor protocols, particularly mechanistic investigation into the promotion stage, has tended to blur the stark distinctions between genotoxic initiating agents and nongenotoxic promoters that constitute the basis for the original two-stage carcinogenesis model (Boutwell 1989). Despite these mechanistic uncertainties, some investigators have described the method as having utility in the development of relative potency values for application in inhalation risk assessments for complex mixtures (Lewtas, Nesnow, and

Albert 1983; Nesnow and Lewtas 1991) Thus, while the mouse skin painting technique does not enjoy universal endorsement as an informative and relevant model for human inhalation exposures of interest, the practical advantages of a medium-term bioassay with a tumor endpoint has led to a continuation of comparative dermal assays of cigarette smoke condensates to the present day. The general utility of the method in certain tobacco-related studies is somewhat constrained by its varying sensitivity to tobacco smoke constituents of interest. The mouse dermal model is relatively insensitive to tobacco-specific nitrosamines, while it responds with high sensitivity to polycyclic aromatic hydrocarbons acting as initiators or complete carcinogens, as well as to certain phenolic fractions active in the promotion phase of tumor development.

Comparative studies of various stocks and strains have indicated that the SENCAR strain exhibits a higher sensitivity in both complete carcinogenesis and in two-stage initiation-promotion protocols than other strains commonly employed in such studies (Slaga and Fischer 1983). The National Toxicology Program similarly demonstrated that the SENCAR mouse was more sensitive than the Swiss (CD-1) and B6C3F1 strains in the initiation-promotion protocol (NTP 1996). The SENCAR stock was selectively bred for sensitivity to skin tumor induction by two-stage tumorigenesis, *i.e.* DMBA initiation/TPA promotion (Boutwell 1964) and has been employed recently in relatively accelerated (30 weeks or less) dermal assays of cigarette smoke condensates

Dermal Carcinogenesis Assays of Tobacco Products

A review of extant *in vivo* investigations employing topical applications of smokeless tobacco products or their extracts is available (Grasso and Mann 1998). These and related laboratory animal experiments employing various means to model human oral exposures to snuff products have been unsuccessful in producing neoplasia in the oral cavity.

Some of the most intensive and informative applications of the mouse skin painting model in research on tobacco smoke carcinogenesis occurred in the 1950s and 1960s in the elegant smoke condensate fractionation studies of Wynder, Hoffmann and colleagues (Wynder and Hoffmann 1964). Significant contribution to the overall tumorigenicity of experimental cigarette smoke condensates was traced to certain weakly acidic and neutral fractions. Rapid developments in smoke analytical chemistry complimented the pursuit of the most active fractions of cigarette smoke particulate matter and the characterization of tumor initiating, promoting, and cocarcinogenic subfractions that serve as points of reference to the present day.

The findings of Wynder and Hoffman set the stage for what was perhaps the largest and most ambitious series of applied carcinogenesis experiments ever conceived, the National Cancer Institute's Smoking and Health program of 1969-1979 (NCI 1977, 1980). Over 150 cigarette variables, selected with the guidance of a Tobacco Working Group of prominent industry, academic and government scientists, were evaluated comparatively as potential affecters of the potency of cigarette smoke condensate as a complete carcinogen when applied at three dose levels, six days per week in lifetime (18-month) skin painting studies in ICR Swiss mice.

It is truly unfortunate that the abrupt termination of the massive NCI project in 1979 precluded the completion, analysis, and final synthesis of the smoke chemistry, *in vitro* toxicity, dermal carcinogenesis, and planned smoke inhalation studies (Gori 2000). However, the available findings from the completed mouse skin painting experiments serve as a valuable data resource that may continue to assist in the design of potential reduced-exposure products beyond those envisioned at the outset of the original research program.

Subsequent to the broad investigations conducted under the NCI Smoking and Health Program, mouse skin painting bioassays were successfully employed in a follow-up evaluation

of a tobacco substitute (Coggins et al. 1982), and to address questions that had been raised in regard to the use of cocoa as a cigarette flavoring/casing ingredient (Roemer and Hackenberg 1990). Gaworski and coworkers (1999) reported the results of a series of SENCAR mouse skin painting bioassays that were intended to assess the potential of combinations of different cigarette flavoring ingredients to affect the tumor-promoting activity of cigarette smoke condensates. No indications of any increase in tumor promoting activity were evident for any of the numerous evaluated ingredients. Despite the persistent uncertainties in regard to the relevance of the mouse skin painting model to human exposure scenarios other than those from direct dermal contact, it appears that some degree of reliance upon the method as a means to compare the initiating and promoting and co-carcinogenic potential of cigarette smoke condensates and condensate fractions will continue into the future.

5. THE FUTURE OF *IN VIVO* TOBACCO TOXICOLOGY

Transgenic animal models

A variety of transgenic test animals having any of a number of engineered genetic alterations are becoming commercially available for evaluation and validation. These animals typically contain genomic alterations that are intended to enhance their sensitivity to biologically active substances and provide insights into toxicological mechanisms. These transgenic test systems may prove to have utility as informative *in vivo* tools to advance our understanding of a variety of classes of compounds and mixtures, including those involved in tobacco and tobacco smoke-related biological events.

In light of the general shortcomings of traditional *in vivo* methods in realistically modeling tobacco-associated cancer development, it is likely that that the initial applications of

transgenic animals in tobacco research will be directed toward cancer-related endpoints. Representative features of such transgenic models include:

- Alteration in genes believed to be related to human cancer development *e.g.* endogenous oncogenes or tumor suppressor genes such as *P53*.
- Genetic modifications believed to be insufficient in themselves to spontaneously produce cancer, yet capable of producing enhanced tumor response;
- Modifications having a potential to predispose the animal to tumor development upon exposure to carcinogens;
- Enhancement of tumor induction processes that may have greater relevance in assessing human cancer risk, such as animals having an inserted human c-Ha-ras gene.

TG:AC mouse model. The TG:AC mouse is representative of the transgenic models that may prove informative in tobacco-related investigations, based upon the knowledge that approximately 30% of human cancers exhibit mutations of the *ras* oncogene. The genome of the TG:AC mouse has been constructed to contain the v-Ha-*ras* oncogene fused to the promoter of the *zeta*-globin gene, which regulates its expression. The transgene is not constitutively expressed in the skin, so that the animal's untreated skin appears identical to the normal skin of the parent, or control, FVB/N mouse strain. While the incidence of spontaneous skin papillomas in untreated transgenic mice is very low, hemizygous or homozygous TG:AC mice treated topically with model skin tumor promoters such as TPA readily develop benign papillomas in as few as 4 to 6 weeks, and a proportion of these may progress to malignancy (Tennant, French, and Spalding 1995).

Briefly summarized, the findings of an evaluation of the TG:AC model have demonstrated its potential as a new tool for toxicological assessment. The dermal papilloma

reporter system offers the convenience of conventional skin painting protocols in terms of its readily scored endpoint; and provision of data on tumor incidence, multiplicity and latency. Additionally, the system responds following oral dosing, and is sensitive in its detection of both genotoxic carcinogens and tumor promoters. Tumor development is accelerated to an extent that papillomas may appear in as little as 5 weeks and final results are available within the 13-week study duration traditionally employed to establish dose levels for conventional lifetime carcinogenesis bioassays. Seven of eight genotoxic and nongenotoxic carcinogens, as well as noncarcinogens, were correctly identified in this evaluation of the TG:AC system. These included benzene, which induced papillomas in 70 percent of the tested animals 10 weeks.

The potential advantages of the TG:AC and other transgenic test systems currently under evaluation are tempered by certain disadvantages, including the current high cost of available transgenic animals (commonly hundreds of dollars per mouse), and the complications of interspecies extrapolation that are common to all *in vivo* models. These factors, which include substantial human/animal differences in the metabolism of tobacco smoke constituents (Tricker 2000) and the difficulties inherent in accurately modeling the unique and variable exposures that characterize human smoking, await resolution by the further development of improved models and methods for the *in vitro* assessment of the toxicology of tobacco products.

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CONDUCTING CLINICAL EVALUATIONS IN HUMAN SMOKERS SWITCHING TO REDUCED-TOXICITY CIGARETTES

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Abstract

Clinical evaluations conducted in human smokers who switch to potentially reduced-toxicity cigarettes (RTCs) can be subdivided into two broad categories – smoke exposure studies and health effects studies. Smoke exposure studies seek to determine if reductions in yield of selected smoke components (assayed by chemical analysis) result in concomitant reductions of the same components in human subject volunteers. A wide variety of smoke components and their metabolites have been studied to determine the exposure level in humans including: nicotine, carbon monoxide, free radicals, urinary mutagens resulting from pyrolysis of tobacco protein, benzene, tobacco-specific nitrosamines, benzo[*a*]pyrene and carbonyls. Health effects studies can be subdivided by the disease risk under evaluation including cancer, cardiovascular disease (atherosclerosis, thrombosis, systemic inflammation, arrhythmias and ischemia) and chronic obstructive pulmonary disease (chronic bronchitis and emphysema). The design of clinical evaluations in human smokers usually consists of taking a baseline measurement while smoking the usual brand of cigarette (or a control cigarette) and then taking the same measurements at sequential time points in the future. Also, a crossover design can be implemented in which measurements of short-term markers or endpoints can be taken at

baseline, while either assigned to control or the experimental RTC, and then after crossing over the original control or experimental groups to enhance statistical power. Human subject volunteers should be properly screened for inappropriate pre-existing medical conditions or conflicts of interest that render them unsuitable as research volunteers. All human subject volunteers should sign an informed consent with all research practices consistent with regulations issued by the Office of Human Research Protection.

Introduction

Before beginning the formal introduction, it is important to define the term "reduced-toxicity" cigarette (RTC) as used within the context of this article. There have been significant and steady reductions in 'tar' yields since the mid-1950s (Wynder and Hoffmann, 1979). These 'tar' reductions have been achieved by several methods including reduced tobacco weight, improved filtration, air dilution and agronomic practices. These modifications also significantly reduced yields of constituents associated with the vapor phase. Therefore, chemical reductions in several compounds classified as known, probable or possible human carcinogens by the International Agency for Research on Cancer (IARC) have already been achieved (Smith *et al*., 1997). However, in today's parlance, the term (RTC) refers to further reductions in the number and concentration of cigarette mainstream aerosol components associated with toxicity beyond that seen in current low 'tar' yield cigarettes as classified by the Federal Trade Commission (FTC) method. Recent examples of RTCs include Premier (RJRT, 1988), Eclipse (Borgerding *et al.*, 1998), and a cigarette containing a novel carbon filter (Bombick, D. *et al.,* 1997).

An extensive epidemiological and clinical literature reports that smokers are at an increased risk for a number of chronic diseases as compared with nonsmokers (U.S. DHHS, 1983; U.S. DHHS, 1984; IARC, 1986). The major disease categories associated with smoking include cancer of the lung and other sites (IARC, 1986), chronic bronchitis (Piquette *et al.*,

2000), emphysema (Kobzik, 1999), atherosclerosis (McGill *et al.*, 1997; Bøttcher and Falk, 1999) and thrombosis (Barbash *et al.*, 1993; Burke *et al.*, 1998; Ottenson *et al.*, 1999). Smokers have been shown to differ from nonsmokers in a number of physiological and biochemical measurements that are plausibly related to the risk of developing the diseases listed above (McKarns *et al.*, 1995; McKarns *et al.*, 1996).

There are at least two basic categories of measurements that can be of interest in a clinical evaluation of smokers switching to an RTC. The first measurement category or type of study is related to exposure (Table 1). In an exposure study, the concentrations of selected mainstream smoke constituents reduced by product design in the RTC aerosol are measured in body fluids (blood, urine, saliva, buccal cell mouthwashes) of human subject volunteers before and after switching to the RTC. [Frequently, a metabolite or reaction product, rather than the original smoke constituent, is measured in the subject's body fluid (Van Vunakis *et al.*, 1987).]

The second type of study is a health effects study (Table 2, Figure 1). In a health effects study, some biochemical or physiological alteration related to the subject's cigarette smoking (*e.g.*, pulmonary inflammation) is measured before and after switching to a reduced-risk cigarette (Rennard *et al.*, in press; Eclipse Expert Panel, 2000). Although, the two types of studies can be viewed as having distinctly different endpoints, in actual practice many protocols will contain both exposure and health-related measurements.

Rationale for the Selection of Study Measurements

Table 1 illustrates a series of tobacco smoke components for which exposure measurements can be determined in human smokers. Depending on the chemical alteration to the RTC mainstream smoke aerosol under study, other smoke component analytes could be added to this list.

As seen in Table 2, there are a number of potential study measurements related to the risk of the various smoking-related diseases. The extensive smoking and health literature going back over five decades (Rodgman *et al.*, 2000; Institute of Medicine, 2001) has described many more potential measurements than those listed in Table 2. The rationale for the selection of the particular measurements itemized in Table 2 is based on several factors including: 1) strength of the relationship between adverse alteration of the risk marker and the disease in question; 2) avoiding redundancy; 3) acceptance of the risk marker among the biomedical community; 4) degree of invasiveness required to make the measurement; and 5) interpretability of the measurement.

With these criteria in mind, some specific comments regarding the disease categories should be made. Currently, predicting the risk of cancer based on human data alone is more problematic than predicting the risk of chronic obstructive pulmonary disease or cardiovascular disease. The underlying technical difficulty is that it is very challenging to sample via biopsy, or image, the large surface area of the lung for the presence of pre-cancerous dysplastic lesions, or even for earlier lesions such as squamous metaplasia (Prager, 2000). Therefore, a weight-of-the-evidence approach can be used to support a decreased risk of cancer (Eclipse Expert Panel) that might include the following: 1) reduced exposure to mutagens and carcinogens under actual human smoking conditions (Smith *et al.*, 1996); 2) reduced *in vitro* genotoxicity of cigarette smoke condensates or whole smoke (Bombick, B. *et al.*, 1998a,b); 3) reduced cytotoxicity of cigarette smoke condensates or whole smoke (Bombick, D. *et al.*, 1996, 1998); and 4) reduced tumorigenicity of cigarette smoke condensate as measured in the mouse dermal promotion assay (Eclipse Expert Panel, 2000). That said, the emergence of increasingly sophisticated and accurate markers of disease risk may make reliance on human data obtained by minimally invasive techniques a distinct possibility (Petricoin *et al.*, 2002).

In addition to the measurements noted in Table 2 regarding COPD, other potentially important endpoints that could be considered include induced sputum samples and 99^{m} technetium diethylene triamine pentaacetic acid (DTPA) lung scans. Induced sputum samples have been omitted because the cell count, cytokine and other measurements tend to display more variability than similar measures taken by bronchoalveolar lavage (Piquette, 2000). However, it should be noted that induced sputum samples are less invasive and less expensive to collect than BAL samples. Similarly, 99^{m} technetium-DTPA lung scans are a good method for determining the degree of vascular permeability of the lung and "leaky" pulmonary vasculature can be associated with pulmonary inflammation (Frampton, 2000). However, while the method is sensitive, it is also expensive and represents a less direct measure of pulmonary inflammation than does BAL.

The paradoxical relationship between cigarette smoking and cardiovascular disease requires some background explanation to elucidate the selection of proximal markers of cardiovascular risk (Table 2). A voluminous literature suggests that the major clinical cardiovascular risk factor associated with active smoking is an increased tendency toward thrombosis (Mueller *et al*., 1992; Barbash *et al*., 1993). This tendency may increase the risk of both myocardial infarction and ischemic stroke, due to occlusion of the carotid arteries (American Heart Association, 1998).

The following points summarize the active smoking literature: 1) In hyperlipidemic populations, *e.g*., the United States, smoking is associated with increased atherosclerosis (Roberts, 1989). 2) The abdominal aorta and the carotid arteries are reportedly affected more than the coronary arteries (McGill, 1988; McGill *et al*., 1997). The average increase in coronary artery atherosclerosis in smokers is approximately 25% (McGill, 1988). In fact, a publication from the Pathologic Determinants of Atherosclerosis in Youth (PDAY) study (McGill *et al*.,

1997), reports "no effect of smoking on the extent of either fatty streaks or raised coronary lesions in the right coronary artery." This result was determined from autopsies of 1079 men and 364 women in the 25 to 34 year age group. 3) Other results from the PDAY study (1990) suggest that the increase in smoking-associated atherosclerosis may be related to inflammatory processes. This suggestion is based on the observation that young smokers with the same blood serum cholesterol levels as age-matched nonsmokers, display increases in abdominal atherosclerosis. 4) The relatively small increase in coronary atherosclerosis is not sufficient to account for the approximately two-fold increase in myocardial infarction seen in heavy smokers (McGill, 1988). 5) Smokers display increases in blood coagulability (McKarns *et al*., 1995). 6) In summary, the combination of modest increases in coronary artery atherosclerosis and increased tendency toward thrombosis is believed to underlie the increased myocardial infarction risk reported in smokers (Molstad, 1991).

Protocol Approval and Conduction

Institutional Review Board Review and Approval

The federal Office of Human Research Protection (OHRP) regulates all research in the US using human subject volunteers in which the participants experience more than "minimal risk" (U.S. DHHS, 2001). According to the Code of Federal Regulations, Title 45, Part 46, effective December 13, 2001, minimal risk "means that the probability and magnitude of harm or discomfort anticipated in the research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physical or psychological examinations or tests."

Human evaluations of smokers switching to RTCs would rarely be considered to be "minimal risk." Therefore, for practical purposes, these evaluations would require institutional review board (IRB) review and approval with all subjects providing informed consent.

Good Clinical Practices

Since the tobacco industry is not currently regulated vis-à-vis testing cigarettes in adult smokers, the decision as to whether to conduct the human clinical evaluation under Good Clinical Practices is usually based on the intended use of the data. However, Good Clinical Practices represents the highest standard of research practice and would generally be acceptable to an agency such as the US Food and Drug Administration (USFDA).

Screening of Potential Research Subjects

Screening of potential research subjects prior to enrollment serves several purposes. First, subjects considering quitting smoking during the duration of the evaluation are excluded on ethical grounds. Second, subjects with financial or personal conflicts of interest are excluded to protect data integrity. Third, subjects for whom participation in the protocol would represent an unacceptable risk are excluded. Frequent pre-existing exclusionary medical conditions include: history of myocardial infarction, stable or unstable angina, uncontrolled diabetes, uncontrolled hypertension, stroke, transient ischemic attack, cancer or serious lung disease. Other exclusions would be use of tobacco products other than cigarettes, insufficient daily smoking amounts, or exposure to occupational or environmental toxins (Figure 2).

Protocol Designs

Linear-Sequential Design (Figure 3)

In a linear-sequential design, each enrolled subject has a set of baseline measurements taken while smoking his or her regular brand cigarette. Each subject then switches to the test

(that is, reduced toxicity) cigarette. Over the designated time period of the evaluation, the same measurements are taken again at regular intervals. In this design, each subject serves as his or her own control, with the data being analyzed by comparing the baseline measurements to the measurements after switching.

The strength of this design is its simplicity and use of each subject as his or her own control. A linear-sequential design has two limitations. First, it does not control for a potential treatment effect. A treatment effect is similar to a placebo effect and can introduce some level of improvement in the subject due to paying attention to the subject or to subject expectation of improvement (Feinstein, 1985). Also, this design does not consider a possible temporal effect. A temporal effect can become more important if the length of the evaluation period spans, for example, different seasons with variable exposures to pollens, woodstoves, fireplaces, *etc*. (Feinstein, 1985).

Parallel Design (Figure 4)

In a parallel design, each enrolled subject has a set of baseline measurements taken while smoking his or her regular brand cigarette. Each subject is then randomized to either Group 1 (test cigarette) or Group 2 (control cigarette). The test group receives the RTC. The control group can either stay on regular brand (un-blinded design), or receive a control cigarette that looks identical to the test cigarette (double blind design).

A double blind parallel design controls for both possible treatment/placebo effects and temporal effects. However, relatively large subject numbers are required to achieve sufficient statistical power (Senn, 1993). The primary statistical comparison is between Group 1 (test cigarette) and Group 2 (control cigarette), although both groups after switching can also be compared with the baseline condition. However, the data analysis might identify a subgroup that responds very favorably to a switch to the RTC.

Crossover Design (Figure 5)

As in the two previous designs, in a crossover design each enrolled subject has a set of baseline measurements taken while smoking regular brand cigarette. Each subject is then randomized to either Group 1 (test cigarette) or Group 2 (control cigarette). The test group receives the RTC and the control cigarette is prepared to resemble the RTC. [Neither the investigators nor the subjects know which cigarette has been assigned.] The time period of the evaluation is divided into two equal parts. In the first half of the trial, Group 1 is assigned the test cigarette and Group 2 the control cigarette. At the halfway point, the two groups switch cigarettes. The baseline measurements are repeated at equally spaced intervals during both halves of the evaluation.

The advantages of the crossover design are that the experiment is conducted double blind, it is statistically more powerful than the parallel designs (Senn, 1993), and controls for temporal effects unlike the sequential design. The disadvantage of a crossover design is the possibility that the subjects will experience two metabolic washout periods for the smoke components from the just previously smoked cigarette instead of just one washout period as in the other two designs. In a health effects study, if the RTC is effective, it may affect the performance of the control in period 2.

Conclusion

Clinical studies conducted in human research volunteers are necessary to establish that reductions in mainstream smoke yield of toxic constituents measured via machine-smoking regimens result in concomitant reductions in human exposure (NCI, 2001). At least two factors underlie the inability to directly extrapolate from yield to exposure. First, individual variation in smoking behavior can be significant and difficult to model even when estimating yield under a variety of machine-smoking conditions (Robinson *et al.,* 1998). Second, interactive effects

among smoke constituents might affect absorption, distribution, metabolism and excretion in unpredictable ways (Lee *et al.* 1993; Lee *et al.*, 1994).

Similarly, health effects studies in humans are required to establish that decreases in exposure to smoke components, and reductions in toxicity as measured by animal - bioassays and *in vitro* tests will result in reduced risk to smokers (Institute of Medicine, 2001). As long-term epidemiological studies following switching to reduced-toxicity cigarettes are not a practical product development tool, clinical studies using proximal biochemical and physiological markers indicative of risk are required. The success of a group of medical school studies on the RTC Eclipse in demonstrating reductions in pulmonary inflammation and respiratory symptoms provides evidence that these cigarettes can have demonstrable clinical effects with modest sample sizes (Eclipse Expert Panel, 2001).

While there are similarities between clinical evaluations in human smokers switching to RTCs and pharmaceutical drug or medical device trials, there are also several important differences. First, the purpose of a traditional clinical trial is to establish the safety and efficacy of a drug or medical device. Since smoking cigarettes possesses inherent risks (Viscusi, 1992a,b), safety cannot be established. Also, since the primary self-reported reasons for smoking cigarettes are enjoyment, stress reduction and job performance enhancement (Eysenck, 1980), defining and demonstrating efficacy is also problematic. Finally, the data from a clinical evaluation of an RTC should be examined to determine whether concomitant changes in other chronic disease risk factors, *e.g.*, diet, might have influenced the results.

Conducting clinical evaluations in human smokers switching to RTCs represents a relatively recent but important development in tobacco research. The success of these early experiments will depend on the appropriate selection of sensitive and relevant measurements and effective design of the protocols.

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Table 1. Selected Mainstream Smoke Constituents Used to Track Exposure

Compound
NOx
Formaldehyde
Acetaldehyde
Acrolein
Acetone
HCN
Isoprene
Benzene
1,3-Butadiene
Styrene
Acrylonitrile
Toluene
Vapor Phase Free Radicals
Phenol
Catechol
Hydroquinone
p,m-Cresol
NNN
NAT
NNK
Benzo[*a*]pyrene
Carbon Monoxide

Table 2. Proximal Markers of Risk.

Disease	Proximal Marker of Risk
Cancer	Urinary mutagenicity; DNA microarray - pulmonary macrophages, bronchial epithelial cells, or blood cells; Genotyping - buccal cells or blood cells; Benzene, B[*a*]P, TSNA's, acrolein metabolites or carbonyl adducts; 8-hydroxydeoxyguanosine in buccal epithelial cells or BAL fluid; DNA damage in alveolar macrophages from BAL fluid.
Chronic obstructive pulmonary disease	Respiratory symptoms; visual bronchitis index; bronchoalveolar lavage; pulmonary function tests; 99^{m} technetium DTPA lung scans.
Atherosclerosis	High LDL; low HDL; high triglycerides.
Thrombosis	Fibrinogen; pro-thrombin time (PT); partial thromboplastin time (PTT); direct platlet aggregation; urinary metabolites of thromboxane/prostacyclin; hematocrit.
Arrhythmia	Ectopic heart beats; heart-rate variability; ventricular or atrial abnormalities.
Ischemia	Carboxyhemoglobin; ST-segment depression (ECG); decreased exercise tolerance.
Systemic inflammation	High sensitivity C-reactive protein; white blood cell count; differential white blood cell count; interleukin-1;interleukin-6; IgG; IgM; tumor necrosis factor a.

Figure 1. Possible Relationship Between Exposure and Health Effects. Flowchart illustrating environmental and genetic interaction required for eliciting a health effect following exposure to toxins.

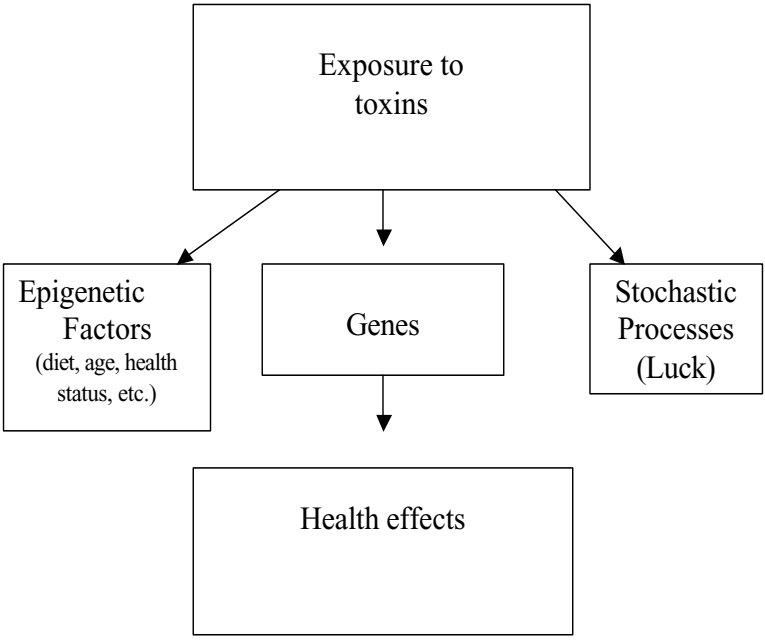


Figure 2. Recruitment and Enrollment of Human Research Volunteers. The process beginning with advertising through randomization is described.

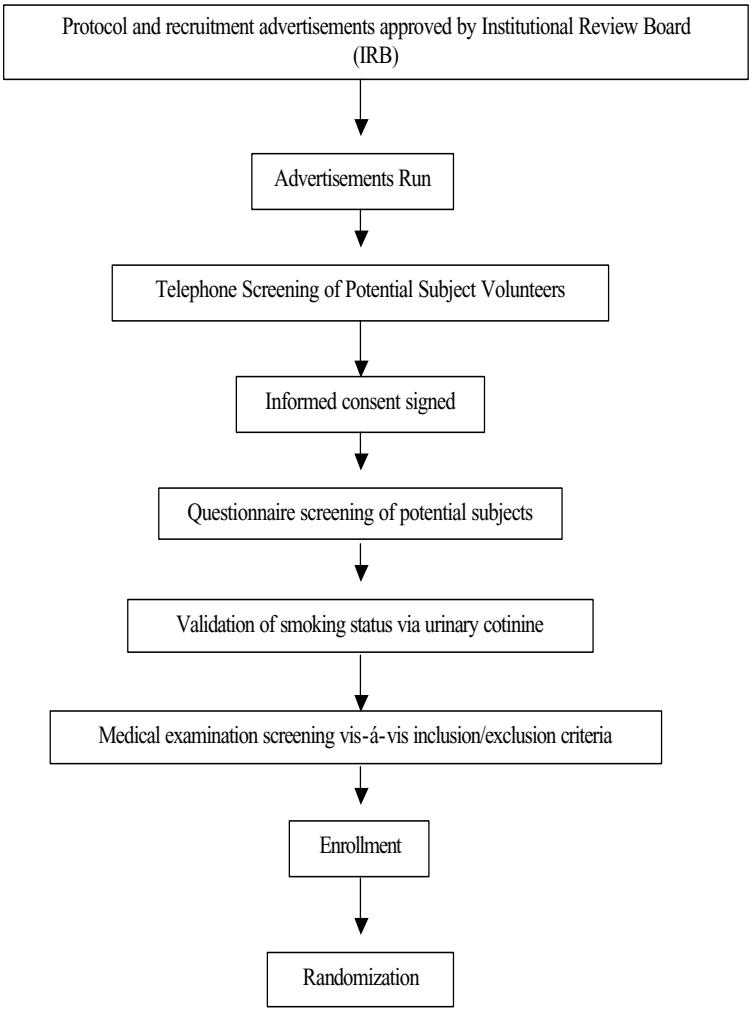


Figure 3. Linear-Sequential Design. Note that the sequential design does not eliminate the possibility of a treatment effect in which the subject improves because of the attention received from the investigators or due to expectation of improvement.

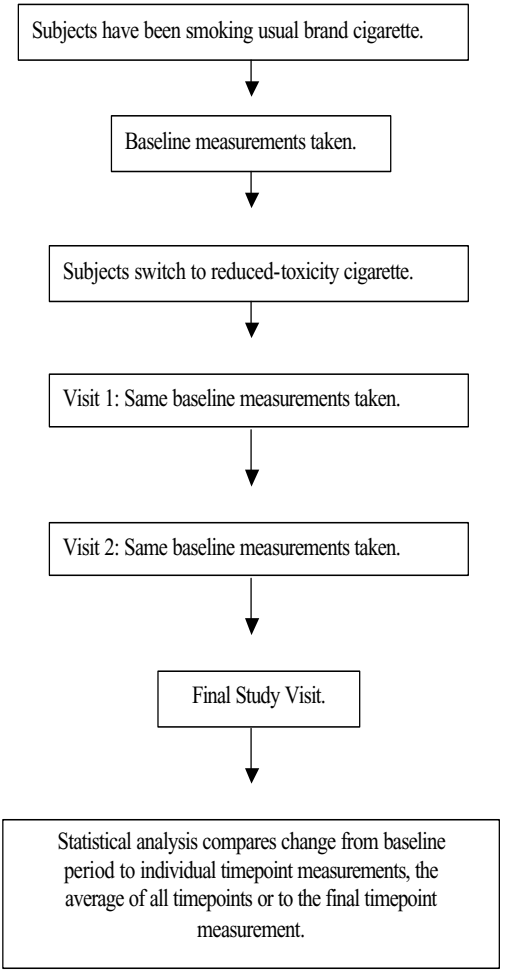


Figure 4. Parallel Design. Note that the parallel design controls for a possible treatment effect and is not strongly affected by a wash-out period following switching cigarettes. However, large subject numbers are required to achieve sufficient statistical power.

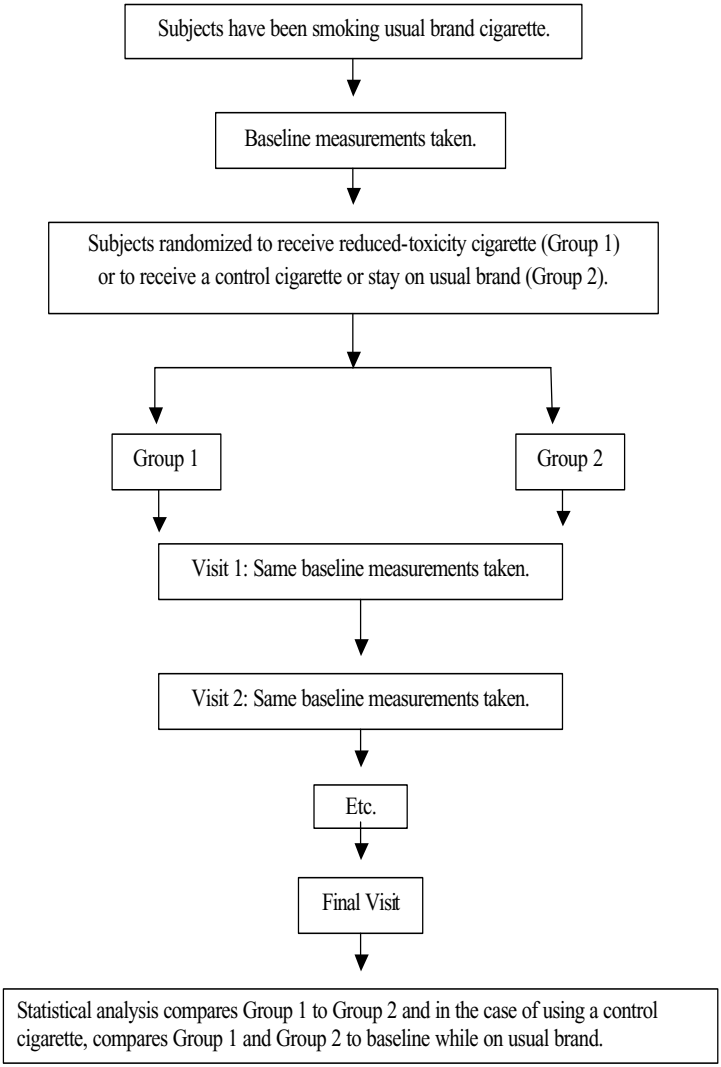
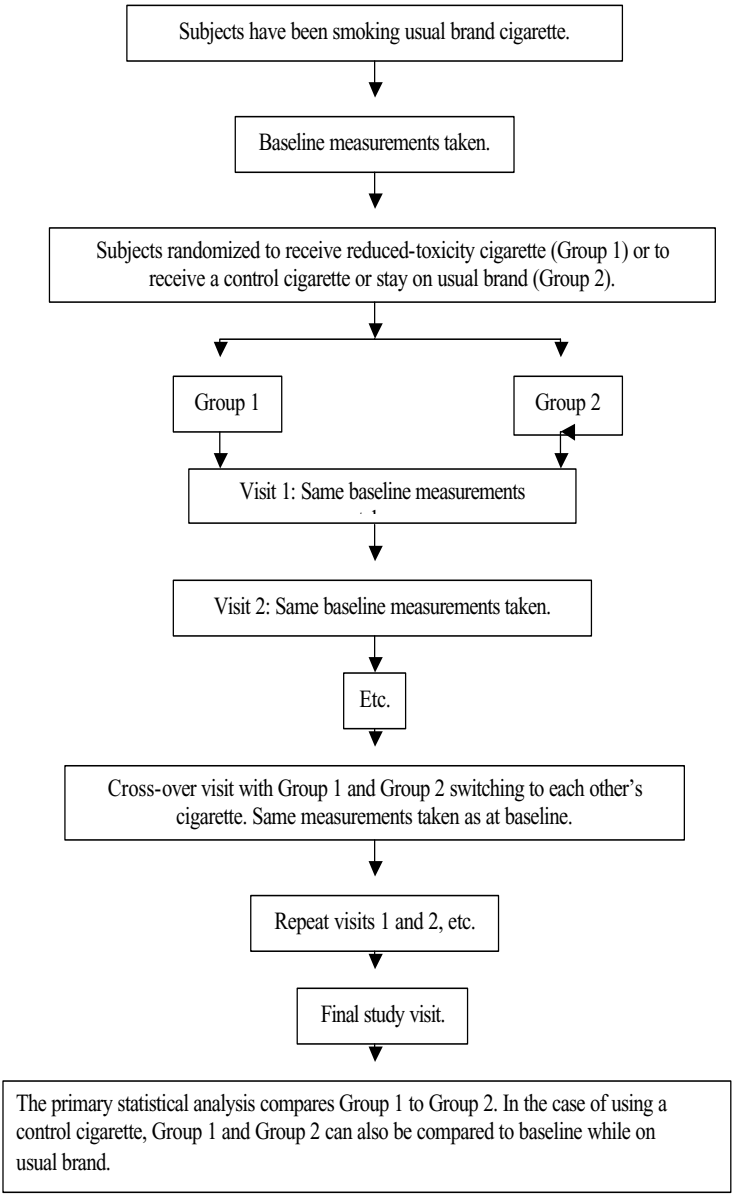


Figure 5. Crossover Design. Note that the cross-over design controls for a possible treatment effect but can be adversely affected by a long wash-out period. A major strength of this design is statistical power.



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