CORESTA *In vitro* Toxicology Task Force

In vitro exposure of cells to Smoke at the Air Liquid Interface

Following the initial objective of the taskforce:

> To prepare a report to cover the rationale and strategy for conducting in vitro testing of tobacco smoke
>
> To identify key procedures based upon internationally recognised guidelines, adapted to accommodate the unique properties of tobacco smoke

The taskforce objectives were widened to conduct a preliminary inter-laboratory study to compare the whole smoke exposure systems under development in a number of laboratories of task force members.

Task force members agreed to evaluate the cytotoxicity, as assessed by neutral red technique, following exposure to the smoke from a range of reference cigarettes. Specifically, laboratories were asked to provide a measure of the contribution of vapour and particulate phases to the cytotoxicity of smoke. Consequently, this report provides a description of the range of systems used in the experimentation and the data produced following measurement of the reference cigarettes used.

Experimental Cigarettes

Kentucky reference cigarette 2R4F

R309 a US grown single grade burley tobacco

R310 a Brazilian grown single grade flue cured tobacco

R311 a 50:50 blend of the tobaccos included in R309 and R310

Table 1. Average smoke chemistries from participating laboratories (mg cig^{-1})

Cigarette	TPM mg cig^{-1}	Nicotine mg cig^{-1}	Water mg cig^{-1}	NFDPM mg cig^{-1}	Puffs
R309 Burley	24.98 ± 1.59	2.71 ± 0.19	3.63 ± 1.17	18.66 ± 1.52	8.75
R310 FC	27.79 ±1.71	2.82 ± 0.09	3.58 ±1.26	21.40 ±1.33	9.93 ±0.39
R311 FC:Burley	26.35 ± 1.31	2.83 ±0.18	3.43 ± 1.05	20.10 ± 1.15	9.97 ± 0.14
2R4F	10.84 ±0.72	0.79 ± 0.05	1.08 ±0.41	8.97 ± 0.56	8.76 ± 0.41

Laboratories

Participating laboratories consisted of:

Altadis	Fleury-les-Aubrais	France
Battelle	Richland	USA
British American Tobacco	Southampton	UK
Imperial Tobacco UK	Hamburg	Germany
Japan Tobacco	Tokyo	Japan
Labstat International	Kitchener	Canada
R.J.Reynolds	Winston Salem	USA

Bioassay Endpoints

All laboratories measured the neutral red (NR) cytotoxicity of the cell cultures using the technique described by the US National Institute of Environmental health Sciences (1). In summary, the cytotoxicity of the smoke or test material is assessed on the basis of the concentration of test material that results in a 50% inhibition of cell viability as assessed by the neutral red uptake.

The lower the concentration that results in a 50% reduction the more cytotoxic the material.

Results

Altadis

The Altadis laboratory used the Cultex (2) air liquid interface exposure system. In summary, for the smoke exposure, cells on porous membranes are brought to the air liquid interface with growth medium underneath and exposed to smoke on the upper surface.

A549 cells were exposed for 40 minutes to a range of smoke dilutions, returned to growth medium followed by a recovery period of 20 hours after which NR cytotoxicity was measured. 3 independent experiments were conducted and the cytotoxicity calculated giving 50% reduction computed for each reference cigarette tested Fig. 1.

The smoke generated from the products were ranked, most cytotoxic R311 (1:1 mixture FC:Burley) > R310 (FC) > R309 (Burley) > 2R4F. However, none of the differences were significant (Fig. 2)

Figure 1. Altadis: A549 Viability versus smoke dilution factor

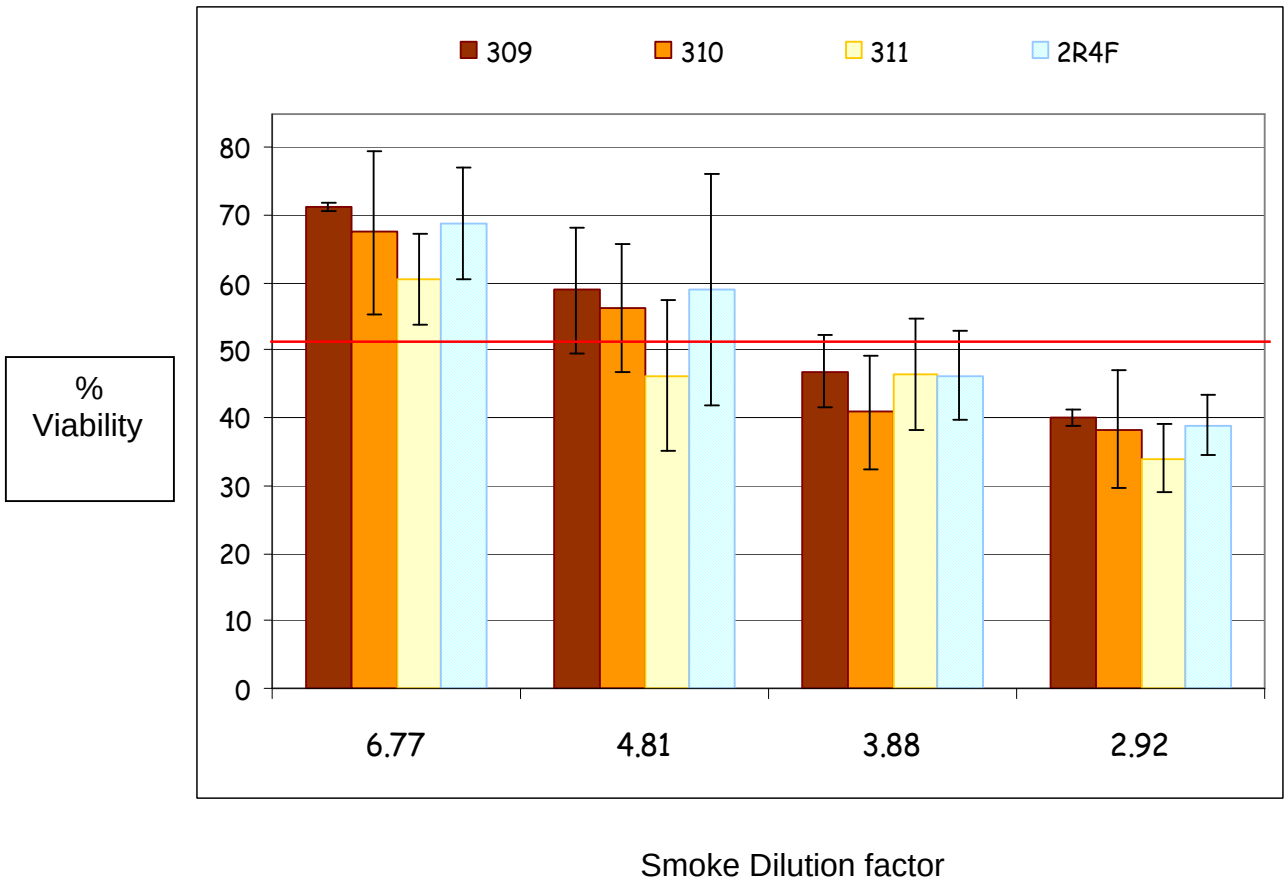
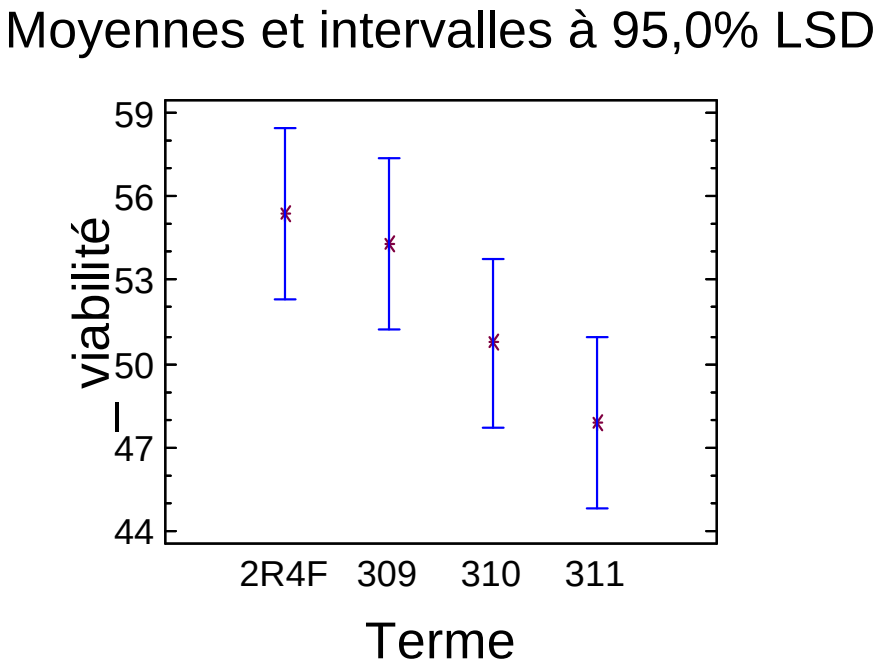


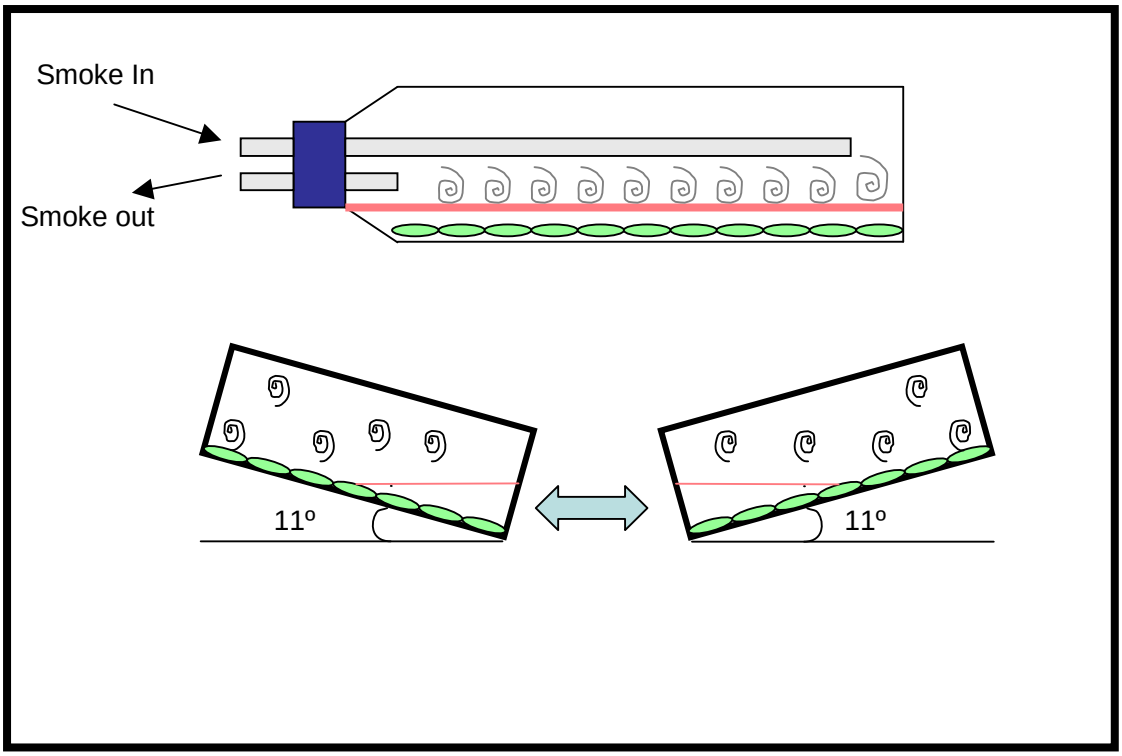
Figure 2. Altadis: Newman-Keuls multiple comparison of smoke responses, most cytotoxic was R311 (1:1 mixture FC:Burley) > R310 (FC) > R309 (Burley) > 2R4F. However, none of the differences were significant



Battelle

The Battelle smoking system consists of BALB/c 3T3 cells maintained in conventional culture flasks on a temperature controlled rocker platform (Fig 3). An AMESA smoke engine was used to supply diluted smoke at a flow rate of 315 ml min^{-1} and the platform operated at 100 oscilations per hour. 4 concentrations per experiments were used and 3 cultures per smoke concentration. Cells were exposed for 1 hour and assayed for cytotoxicity 24 hours after smoke exposure.

Figure 3. Diagramatic of whole smoke exposure system.



The Inhibitory concentrations of smoke or vapour phase equivalents resulting in a 50% inhibition of cell viability are illustrated in table 2.

The data were assessed as a cytotoxic ranking of 2R4F > R310 FC > R309 Burley with a contribution of 70 to 90 % from the vapour phase components.

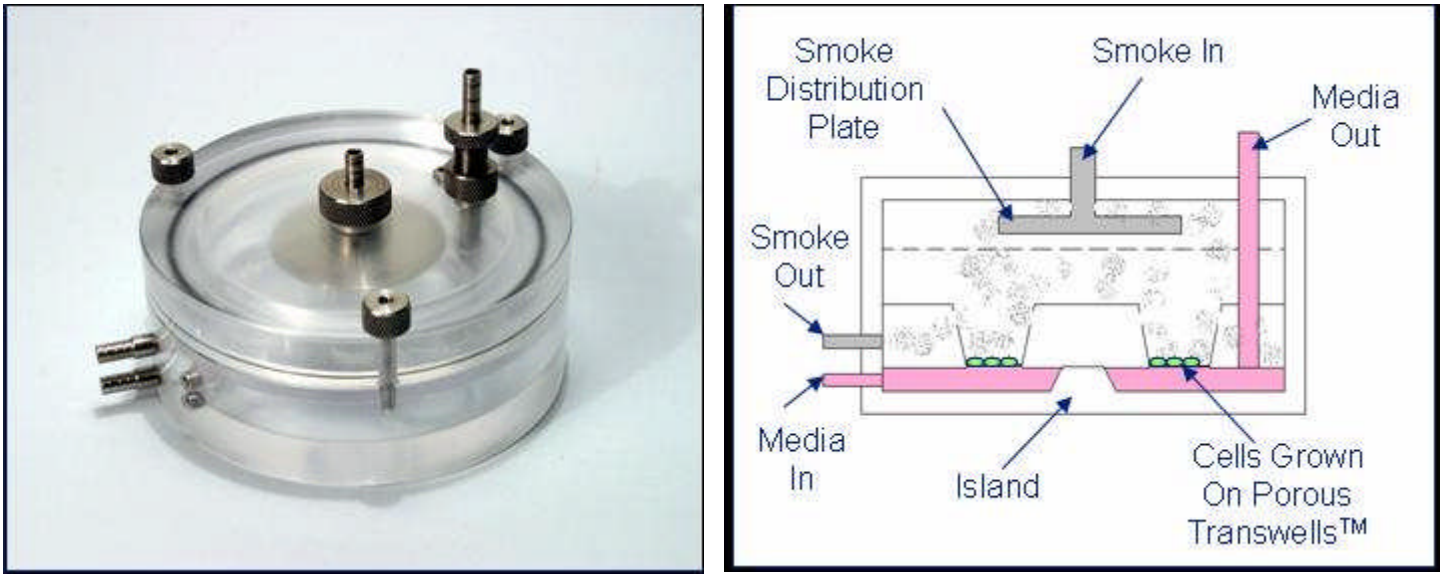
Table 2. Battelle: Inhibitory concentrations resulting in a 50% inhibition of cell viability (µg Litre^{-1} wet TPM or equivalents)

Cigarette	Exp. 1	Exp. 2	Mean
2R4F Smoke	30.1	31.6	30.9
Vapour	38.5	40.0	39.3
R309 Burley Smoke	90.5	90.8	90.7
R309 Burley Vapour	88.9	99.2	94.1
R310 FC Smoke	57.4	58.6	58.0
R310 FC Vapour	72.4	72.0	72.2

British American Tobacco

The BAT system is described in reference 3.

Figure 4. BAT whole smoke exposure chambers



Diluted smoke is generated by a Borgwaldt RM200S smoking engine and cells on porous membranes are exposed from the upper surface as illustrated in figure 4. whilst at the same time supplied from below with tissue culture media. A human lung epithelial cell line H292 was exposed for 30 minutes and neutral red cytotoxicity measured 20 hours after exposure to smoke. The data is from 3 independent experiments and the smoke concentration is recorded as a dilution factor resulting in a 50% inhibition of cell viability, larger numbers indicate a more cytotoxic smoke.

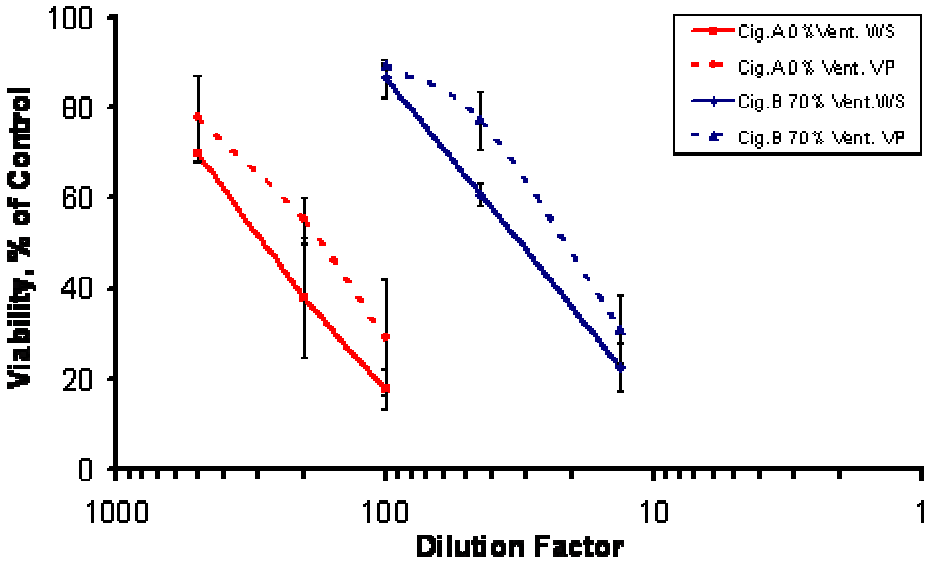
The cytotoxicities of the smokes were ranked as illustrated in table 3. Flue cured was most cytotoxic followed by 2R4F, and burley was least cytotoxic. However, these differences were not statistically significant when evaluated by t-testing.

Table 3. BAT - Smoke dilutions producing 50% inhibition of cell viability

Cigarette	Dilution to give Inhibitory concentration 50%
R310 Flue Cured	108
2R4F	94
R309 Burley	87

For the vapour phase experiments, a Cambridge filter was included between the cigarette and the exposure chamber, the data is illustrated in figure 5. The shift in the dose response curve for vapour phase in comparison to whole smoke was calculated with the vapour phase accounting for 80% - 90% of the response.

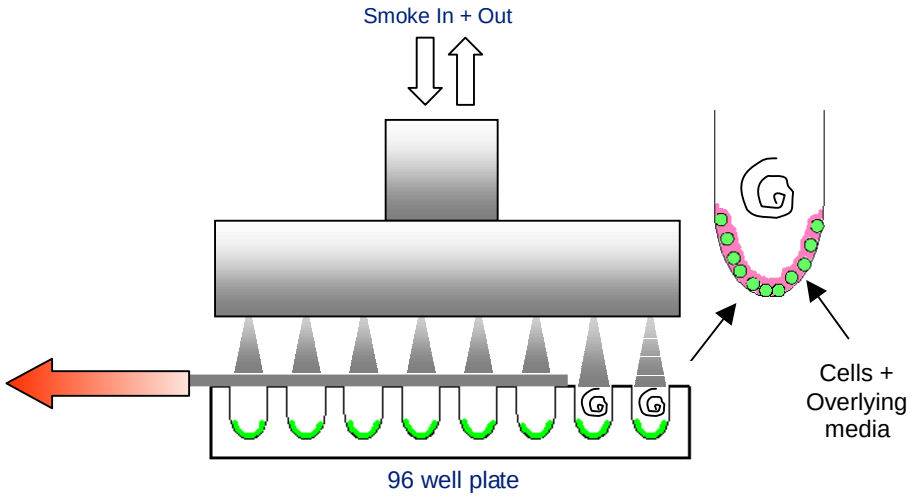
Figure 5. Responses for cigarettes with either 0% or 70% ventilation illustrating the contribution from both whole smoke or vapour phase.



Imperial Tobacco UK (IT)

The IT system uses a standard 96 well cell culture plate with 35 ml smoke 'injected' into each of the 96 wells Fig 6. A Hep-G2 human liver cell line is used, growth medium is poured off the plates and smoke passed puff by puff over the cells for 30 minutes. The cells are then returned to culture under growth medium for a further 65 hours and then assayed for neutral red cytotoxicity.

Figure 6. Imperial Tobacco : Fresh smoke exposure system (whole smoke or vapour phase)



The puff specific concentrations resulting in a 50% inhibition in cell viability are illustrated in Figure 7, indicating most cytotoxic R310 (FC) >R311 (1:1) >R309 Burley >2R4F. When a Cambridge filter was placed between the cigarette and the exposure system, there is a 10% - 25% contribution from the vapour phase to the cytotoxicity of smoke, the remainder coming from the particulate phase (Figure 8).

Figure 7. Imperial Tobacco – Relative cytotoxicities of smoke from the reference cigarettes expressed as number of puffs reducing cell viability by 50%

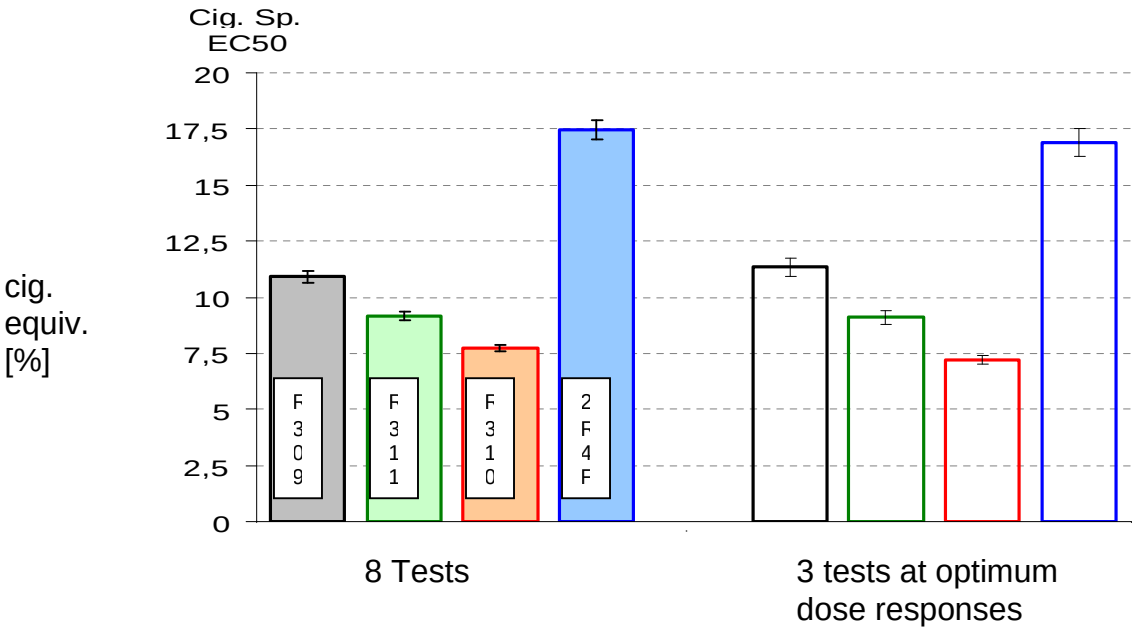
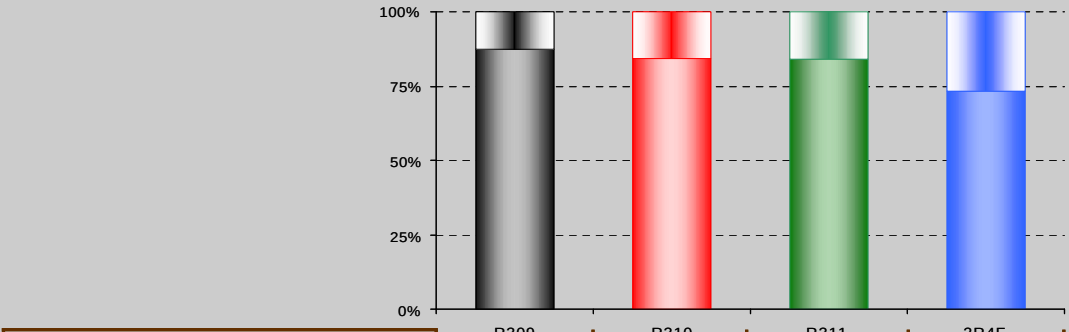


Figure 8. Imperial Tobacco. Proportion of the cytotoxic response contributed by vapour phase and particulate phase components of tobacco smoke.



Cigarette	R309 Burley	R310 Flue cured	R311 1:1 Blend	2R4F 2R4F
% of Vapour Phase toxicity of Whole Smoke toxicity	12.5	15.7	16.0	26.6
ISO-TPM [mg/cig]	25.5	29.2	26.9	11.9
mg / Cigarette	895	944	956	1060
Puffs / Cig.	7.3	8.5	8.4	7.7

Japan Tobacco (JT)

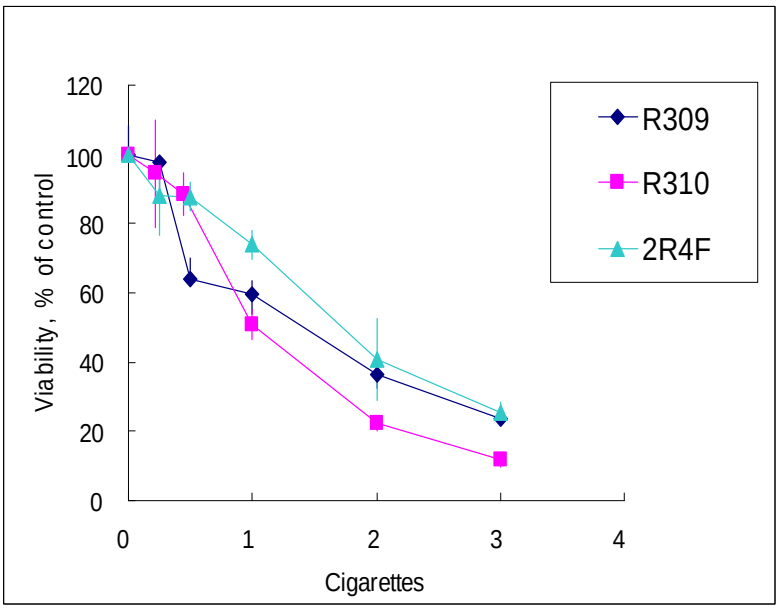
The JT group used the CULTEX^{TM} air liquid interface exposure system with A549 cells maintained on Transwell^{TM} membranes. The cells were subject to 2 to 30 minute exposures of diluted smoke from a 35ml puff separated by 1 minutes then returned to submersion culture for 18 hours before measuring the cytotoxicity by the neutral red accumulation technique. The data for the reference products are set out in Figure 9 and when measured on a per cigarette basis, the cytotoxic ranking was (no statistical computation):

R310 (FC) > R309 (Burley) > 2R4F (Table 4)

Table 4. Japan Tobacco. Cigarettes giving 50% inhibition of NR cell viability

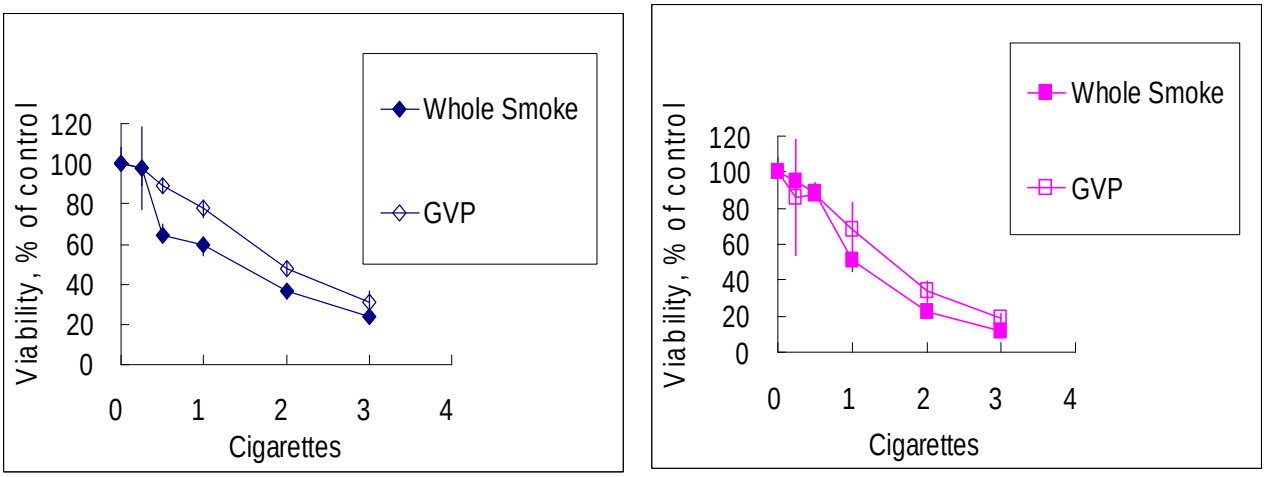
Cigarette	Cigarettes giving 50% reduction in NR cytotoxicity
R310 Flue cured	1.06
R309 Burley	1.44
2R4F	1.65

Figure 9. Japan Tobacco plots of NR dose responses for the 3 reference cigarettes.



The response from the vapour phase vs whole smoke is illustrated in Figure 10, with an estimation of a 66 – 76% contribution of vapour phase to the cytotoxicity of the whole smoke.

Figure 10. Japan Tobacco. Cytotoxicities of whole smoke versus smoke vapour phase (GVP)



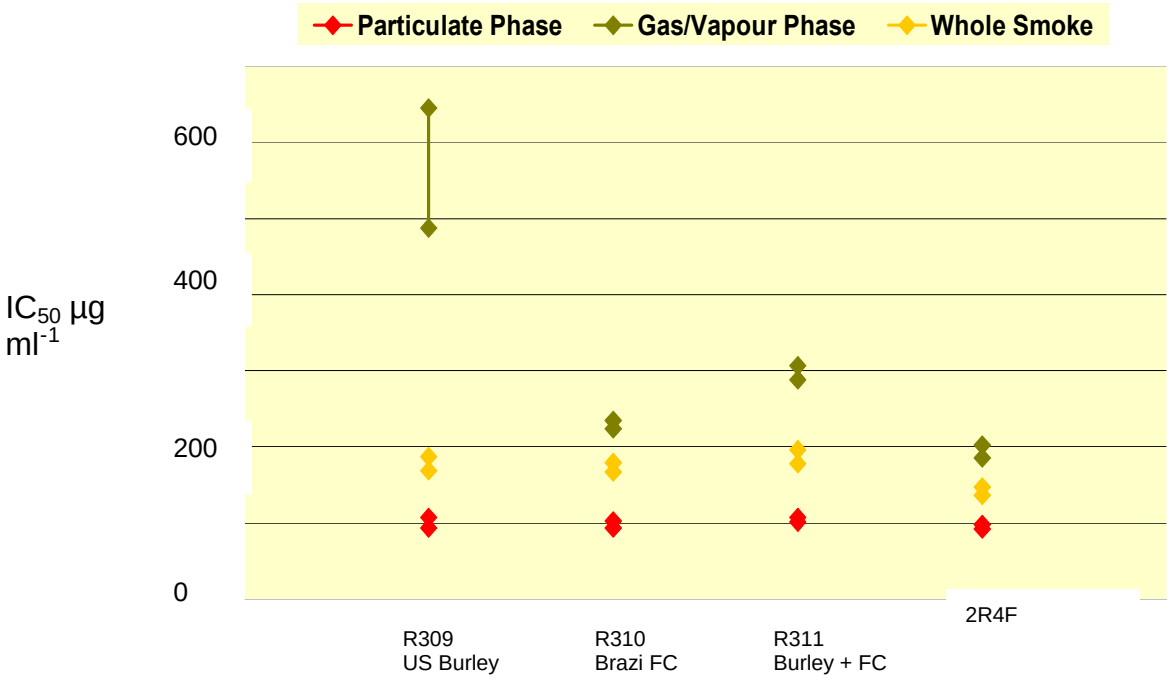
Labstat International

Labstat assessed the cytotoxicities of the smoke from the reference cigarettes using the methodology set out in the Health Canada guidelines (4). In summary, using an ISO smoking regimen, particulate matter (TPM) is collected on Cambridge filter pads whilst simultaneously collecting the gas vapour phase in phosphate buffered saline. TPM is then extracted into dimethyl sulphoxide (DMSO) (10mg TPM ml^{-1}) whilst the gas vapour phase is adjusted to give a 10 mg ml^{-1} TPM equivalent in phosphate buffered saline. For the whole smoke solution the DMSO and gas vapour phase solution are combined at a 1:1 ratio.

A CHO cell line was exposed to a concentration range of smoke solutions for 24 hours followed by the neutral red cytotoxicity procedure. Note there is no recovery phase between the treatment with smoke solution and the measurement of cytotoxicity.

The plot of smoke concentrations inhibiting cell viability by 50% are illustrated in Figure 11.

Figure 11. Labstat International cytotoxicities of test products

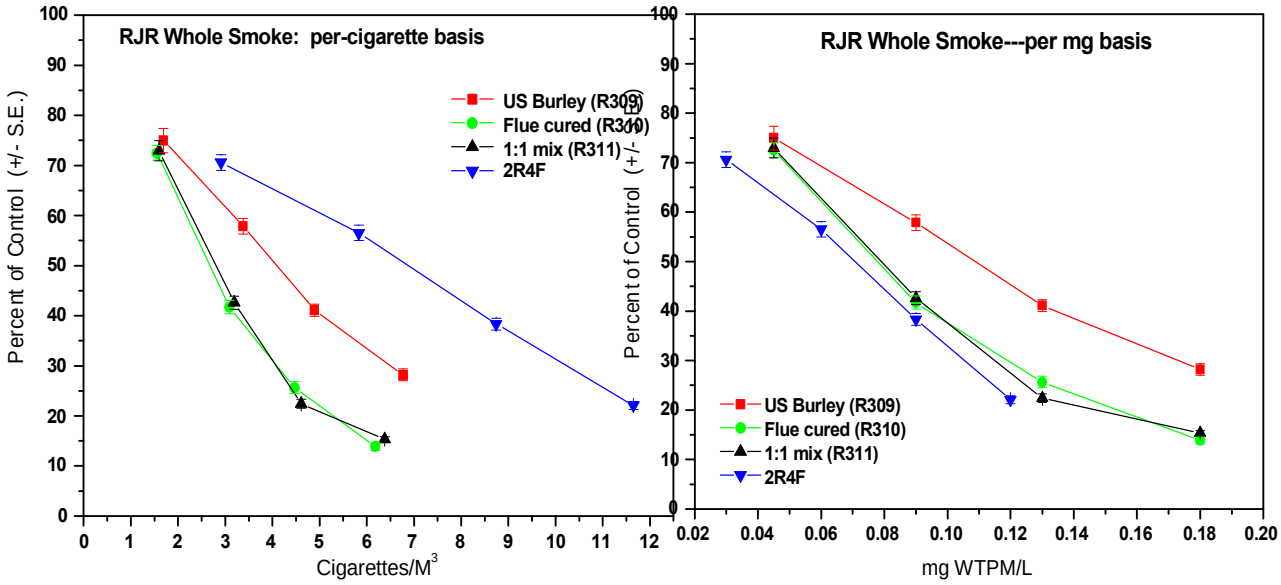


These data were subject to analysis of variance and student-Newman-Keul Multiple Comparison tests which indicated that the whole smoke from 2R4F was significantly more cytotoxic than that from the other reference products which could not be differentiated in activity.

R. J. Reynolds

The system used is in principle the same as that used by the Battelle group a rocking platform maintained at 37°C on which cell culture flasks are placed and supplied with diluted smoke (Bombick, 1997).CHO cells were used as the target and experiments conducted on 3 separate occasions. The data (Fig. 12) were log transformed and statistical analysis carried out using a pairwise T-test from ANOVA, with Bonferroni adjustment. The product ranked as most cytotoxic was R310 (FC) > R311 (1:1 mix) > R309 Burley > 2R4F. The difference between the R310 FC and R309 burley was statistically significant, whilst the 2R4F smoke was significantly less cytotoxic that the smoke from the other reference cigarettes.

Figure 12. R. J. Reynolds. Data expressed per mg wet TPM and per cigarette.



Summary and Conclusions

- All laboratories were able to measure the whole smoke cytotoxicity of the reference cigarettes provided.

- The differences in the cytotoxicity of the reference products tested were minimal with occasional significant differences between the smoke cytotoxicities.

- All laboratories conducting exposure at the air liquid interface, ranked the smoke from flue cure cigarettes as marginally more cytotoxic than the smoke from the all burley cigarette

- For both exposure on Transwell^{TM} membranes and in cell culture flasks the vapour phase contribution to whole smoke cytotoxicity was in the region of 70% to 80%.

- The cell line used to perform the experiments did not have a significant effect on the data patterns produced nor did minor protocol variants.

Overall, the whole smoke exposure systems produce remarkably similar data sets and form a platform for the Industry to be able to investigate the *in vitro* toxicology of vapour phase of tobacco smoke.

Table 5. Summary table of basic parameters of the exposure systems and cytotoxic rankings of the smokes.

Group	Cell Line	Exp. Time (min)	Post exp (hrs)	Comparison basis	Rank Cytotoxicity	% VP
Altadis	**A549**	40	20	Cigarette	1:1 ≥ FC ≥ USB ≥ 2R4F	
BAT	**H292**	30	20	Cigarette	FC ≥ 2R4F ≥ USB	80-90
ITL	**Hep-G-2**	30	65	Cigarette	FC > 1:1 > USB > 2R4F	10-25
JT	**A549**	2 - 30	20	Cigarette	FC > USB > 2R4F	66-76
Labstat	**CHO**	24 hr	--	TPM + GVP	2R4F > FC = USB = 1:1	
RJR	**CHO**	60	24	TPM Cigarette	2R4F ≥ FC = 1:1 > USB FC = 1:1 > USB > 2R4F	
Battelle	**3T3**	60	24	TPM	2R4F > FC > USB	70-90

References

1. US NIH publication No: 07-4519 In vitro cytotoxicity test method for estimating starting doses for acute oral systemic toxicity tests. http://ntp-apps.niehs.nih.gov/iccvampb/searchDoc.cfm

2. Aufderheide M., Knebel J.W., Ritter D. An improved *in vitro* model for testing the pulmonary toxicity of complex mixtures such as cigarette smoke. Exp. Toxicol. Pathol. 55 (1) 51-57, 2003

3. Phillips J., Kluss B., Richter A. and E. D. Massey Exposure of bronchial epithelial cells to whole cigarette smoke: assessment of cellular responses. ATLA 33, 239-248, 2005.

4. Health Canada Official method T-502 Neutral Red Uptake Assay for Mainstream Tobacco Smoke

5. Bombick D.W., Ayres P.H. and D.J. Doolittle. Cytotoxicity Assessment of whole smoke and vapor phase of mainstream and sidestream cigarette smoke from three Kentucky Reference cigarettes. Tox Methods, 7: 177-190, 1997.