Endotoxin, unidentified material, and low amounts of defined toxicants are confounding factors when using the air pollution Standard Reference Material (SRM) 2786 in biological experiments.

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Abstract
The National Institute of Standards and Technology (NIST) provides the reference sample SRM 2786 for use in analytical studies of particulate air pollution. The sample originated from an atypical source and possibly contains materials that are unrelated to air pollution. Investigators conducting studies with SRM 2786 should note the relatively low amounts of toxic components listed in the certificate of analysis as well as a large amount of uncharacterized material. The presence of endotoxin in SRM 2786 is reported here. Endotoxin and other uncharacterized material in SRM 2786 may influence results and conclusions for biological studies using this reference material.

Introduction
Airborne particulate matter (PM) is complex and variable in its composition and has been linked to adverse health effects (1). Consequently, particulate matter of 10 microns (PM10) and 2.5 microns (PM 2.5) in the air are monitored to protect respiratory health. The US EPA and the World Health Organization have established standards of 12 ug/m^3 and 5 ug/m^3, respectively, as acceptable levels of PM2.5 in air (2, 3). Airborne PM is regulated by its size rather than its composition and toxicological properties. For example, PM2.5 in the air might come from an anthropogenic source such as the exhaust from a diesel engine, or natural sources such as wind-blown soils and biological agents such as bacteria and viruses. Particulates from these different sources produce different effects when inhaled into the lungs, but they all are considered PM 2.5 for the purpose of regulation.

A particulate matter sample, SRM 2786, was prepared and partially characterized by the National Institute of Standards and Technology (NIST) of the USA (4). Rather than being directly isolated on standard air sampling media, this material was obtained from “an air intake filtration system of a major exhibition center in Prague, Czech Republic.” This source of material is problematic because the air filter type, usage time, and amount of air filtered are unspecified. Commercial air filters that are in place for extended periods of time can be contaminated with non-airborne material and may provide a source for microbial growth. Thus, SRM 2786 may not be representative of actual PM 2.5 air pollution.

SRM 2786 was not specifically prepared or characterized for use in biological or toxicological experiments. The material from the commercial air filter was fractionated

by size to < 4 um and then partially characterized for numerous elements and organic molecules as described in its certificate of analysis (4). A summary of these analytical categories and their amounts are shown in Table 1 below. Low amounts of potentially toxic metals and organics were measured in this sample. Seventy-eight percent of the sample (by weight) is unknown, and about 20% consists of elements (mostly sodium, magnesium, calcium, iron, aluminum, and chlorine). Some of the metal components of SRM 2786 will not remain PM2.5 when placed in aqueous or biological environments since they likely exist as soluble salts. Additionally, SRM 2786 was characterized for specific polycyclic aromatic hydrocarbons (PAHs), nitro-PAHs (N-PAHs), polybrominated diphenyl ethers (PBDEs), chlorinated dioxins and dibenzofurans (Dioxins), hexabromocyclododecane isomers (HBCDs), anhydrosugars (levoglucosan, mannosan, glactosan) and moisture content. The polycyclic aromatics and halogenated organic compounds collectively constitute <0.1% of SRM 2786.

Table 1. Summary of the analyte groups in SRM 2786 (g/kg)*.

Elements	PAHs	N-PAHs	PBDEs	Dioxins	HBCDs	Anhydrosugars	Moisture	Uncharacterized
203	0.1	0.0014	0.00025	0.000013	0.0006	0.6	17	~780

*Summarized from the SRM 2786 certificate of analysis (4).

Hill, *et al* used SRM 2786 as a prototypical PM2.5 air pollution source to induce inflammation in a murine model of lung cancer (5). While the authors assumed the inflammatory tumor-promoting effects were due to the particulate nature of the exposure, no unsized control material was used for purposes of comparison. Consequently, it is unclear if the particulate nature of SRM 2786 is required for the inflammatory effects. Ideally, the dose amount and route of exposure in animal studies should be physiologically relevant for purposes of extrapolation to people. The experiments reported by Hill, *et al* did not include a rationale for the dosing protocol of 5 or 50 ug per treatment of 9 tracheal instillations of SRM 2786 over 21 days. Tracheal instillation is useful in experiments because it delivers a known dose but is less physiologically relevant than exposure through normal breathing mechanisms. Tracheal instillation of SRM 2786 circumvented the potential for particulate interactions in the upper respiratory tract and delivered the entire dose immediately to the lung. This resulted in a greater lung concentration than if the exposure was given by breathing over time. Table 2 relates the airborne particulate exposure using physiological parameters of murine breath rate and volume (6, 7) for two levels of PM2.5 in the air that would be needed to achieve the doses of SRM 2786 instilled into the lungs. The doses of SRM 2786 used, assuming 100% delivery, were far above what would be encountered through normal breathing. The high dosage makes it difficult to relate the Hill, *et al* study to human exposures and health effects from PM 2.5 air pollution.

Table 2. Inhaled PM2.5 doses by breathing compared to SRM 2786 intratracheal instilled doses.

Hypothetical PM2.5 in air (ug/m^3)	Air inhaled by mouse (m^3/d)	PM2.5 deposited in lungs (ug/d)	Total SRM 2786 instilled (ug)	Days air breathing to reach total SRM 2786 dose
12*	0.03	0.36	45 (5 ug x 9)	125
12	0.03	0.36	450 (50 ug x 9)	1250
120**	0.03	3.6	45 (5 ug x 9)	12.5
120	0.03	3.6	450 (50 ug x 9)	125

* US Environmental Protection Agency air quality standard for PM2.5
** A hypothetical extreme airborne PM2.5 level

Research methods and results

Since SRM 2786 contains very low amounts of measured genotoxic compounds and elements and a large amount of uncharacterized material, it becomes important to identify what component(s) produced the tumor-promoting inflammation described by Hill, et al. One possibility is that SRM 2786 contains endotoxin. To test this idea, one ml of sterile water was added to approximately 100 mg SRM 2786 to produce a final concentration of 100 mg/ml. The sample was vortexed extensively to dissolve as much of the material as possible. A Pierce chromogenic endotoxin test kit (ThermoFisher A39552) was used as directed to detect the presence of endotoxin. After the reaction was stopped, the assay plate was allowed to stand for 1 hour and then 150 ul of the assay supernatant was pipetted into a new 96-well plate. This transfer eliminated the interfering sedimented material at the bottom of the assay plate. The SRM 2786 sample was incubated with the chromogenic substrate alone (no *Limulus* enzyme) to verify that there was no spontaneous activity. Spectrophotometric absorbance readings were taken on the wells of the new plate at 405 nm to detect nitroanilide, the indirect product of the bioassay that indicated the presence of endotoxin. Known amounts of endotoxin were used to construct a standard curve to relate the amount of endotoxin present in SRM 2786.

Table 3. Endotoxin assay and levels in SRM 2786

Endotoxin units, standard curve	Abs. 405 nm (+/- S.D.)	SRM 2786 (ug)	Abs. 405 nm (+/- S.D.)	Endotoxin (units/ug SRM 2786)
1	0.95 (0.05)	50	Out of range	-
0.33	0.78 (0.11)	16.7	0.94 (0.04)	~ 0.06
0.1	0.54 (0.05)	5.5	0.74 (0.03)	~ 0.06
0	0 (background subtracted)	-	-	-

Based upon extrapolation from the standard curve, the SRM 2786 sample contains approximately 0.06 endotoxin units per ug. Each dose of 5 and 50 ug would deliver approximately 0.3 and 3 units of endotoxin in the lung instillation experiments. Based on previous studies, these amounts of endotoxin would be expected to produce inflammatory responses and cytokine expression in the lungs (8-9).

The SRM2786 sample was also tested for viable microorganisms. Five mg in water was spread onto tryptic soy agar plates and compared with a vehicle control (water alone). Plates were incubated at 22 and 37 ^{o}C for 5 days. No microbial growth was detected from the SRM 2786 material. Thus, SRM 2786 may lack viable microbes, but it contains considerable endotoxin. Endotoxin can be present in the air as a natural microbial product or may have come from microbial growth on the original air filter that provided the starting material for SRM 2786. The latter possibility becomes probable if the air filter was used for an extended period in an environment that allowed exposure to sufficient humidity and other factors that favored microbial growth.

Summary

SRM 2786 is a complex material that was partially characterized for specific chemical components. It is suitable as a reference standard for analytical studies of PM2.5 but should be used with caution in biological systems since it contains endotoxin and other uncharacterized material. No experiments have been performed to discern whether the particulate nature of SRM 2786 is required for its biological effects or if it has mutagenic activity. Environmental studies of PM2.5 tend to focus on anthropogenic air pollution sources which are regulatable. Indeed, pollution controls have resulted in a considerable decline in air pollutants in the US and better respiratory health. Deployment of better emission controls, improved vehicle fuel efficiency, and the switch to electrical energy sources will continue to reduce anthropogenic air pollutants in the future. Endotoxin, microbes, and other natural materials can be abundant bioactive components of PM2.5 and produce strong inflammatory responses (8-10). Natural agents as well as anthropogenic ones should be accounted for when conducting studies on health effects of air pollution.

References

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