BIOASSAY

MEASUREMENT AND ANALYSIS OF THE BIOLOGICAL ACTIVITY OF $PM_{2.5}$ SAMPLES

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MEASUREMENT AND ANALYSIS OF THE BIOLOGICAL ACTIVITY OF $PM_{2.5}$ SAMPLES

1. PURPOSE AND APPLICABILITY

This OP contains the protocol for performing measurements of the biological activity of $PM_{2.5}$ samples as measured by the ability of particle samples to induce the secretion of cytokines and reactive oxygen species (ROS) by cultured alveolar macrophage and airway epithelial cells. This is an evaluation version of an anticipated standard operating procedure (SOP), which will result from experiences with this OP. Due to this fact, this OP is subject to changes. Every addition/change to this OP will be added as an Appendix during this study.

2. DEFINITIONS

TNF_{α} TUMOR NECROSIS FACTOR
IL-6 INTERLEUKIN 6
IL-8 INTERLEUKIN 8
GM-CSF GRANULOCYTE MACROPHAGE-COLONY STIMULATING FACTOR
ROS REACTIVE OXYGEN SPECIES
LPS LIPOPOLYSACHARRIDE

3. REFERENCES

Chen, L.C., Su, W.C., Qu, Q., Cheng, T.J., Chan, C.C. and Hwang, J.S. (1999). PM Meeting Abstract.

Ensor, J.E., Crawford, K.K. and Hasday, J.D. (1995) Am. J. Physiol. 269: C1140-1146.

Imrich, A., Ning, Y.Y., Koziel, H., Coull, B. and Kobzik, L. (1999). PM Meeting Abstract.

Veronesi, B., Oortgiesen, M., Carter, J.D. and Devlin, R.B. (1999) Toxicol. Appl. Pharm. 154: 106-115.

4. DISCUSSION

What should this section include????

5. RESPONSIBILITIES

List of personnel and individual responsibilities???

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6. EQUIPMENT AND MATERIALS

6.1 Equipment

Incubator, Microplate reader, Cytofluor
(Will expand to include make and model numbers)

6.2 Materials

(Expand to include sources and catalog numbers?)

Cell lines:
RAW 264.7 Mouse macrophage cell line (ATCC # TIB-71)
A549 Human alveolar Type II cell line (ATCC # CCL-185)

Media:
RPMI
Ham's F12K

Cell culture supplies:
LPS (E. coli serotype 0127:B8)
Penicillin
Streptomycin
Fetal Bovine Serum
Glutamine
Sodium pyruvate
HEPES buffer
Sodium bicarbonate
Polymyxin B

Cytokine ELIZA assay:kits
Lactate Dehydrogenase assay reagents
Lymulus polyphenus amebocyte assay
Trypan blue
Crystal violet
Dichlorodihydrofluorescein diacetate
Phorbol myristate acetate (PMA)

Particles:
SRM 1649 Standard Urban Air Particles
Titanium dioxide

7. INSTRUMENT MAINTENANCE PROCEDURES

7.1 Routine maintenance procedures

All Maintenance procedures must be recorded in the field form.

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Instrument specific maintenance procedures will be added

7.2 Handling of persistent instrumental deviations

If any adjustment of the operating parameters yields no sufficient result (i.e. normal operating conditions can not be achieved), personal doing the routine maintenance is requested to report the problem and actions undertaken to solve it to an experienced operator as soon as possible.

8. ANALYTICAL PROCEDURES

8.1 Collection, storage and preparation of $PM_{2.5}$ samples

8.1a *Collection of $PM_{2.5}$ samples:* See SOP for semi-continuous elemental aerosol sampler (SEAS)

8.1b *Storage of $PM_{2.5}$ samples*: Collected $PM_{2.5}$ fractions will initially be placed in a –20°C freezer in the trailer facility for storage for no longer that 48 hr, at which the samples will be transferred to the UM,B laboratories and stored in a –70°C freezer until analyzed.

8.1c *Preparation of $PM_{2.5}$ samples for bioassay measurements*: $PM_{2.5}$ samples will be lyophilized to concentrate the sample and weighed to obtain a dry weight measure. At the time of analysis, each sample will be resuspended in media containing penicillin (50 U/ml) and streptomycin (50 µg/ml) at a concentration of 1 mg/ml and sonicated for 20 minutes in an ultrasonic water bath.

8.2 Maintenance of cell cultures

8.2a *RAW 264.7 mouse macrophage cell line:* RAW 264.7 mouse macrophage cells (American Type Culture Collection, Rockville, MD; ATCC # TIB-71) will be maintained in RPMI 1640 (Mediatech, Fairfax, VA) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, l0 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (GIBCO BRL, Gaithersburg, MD), pH 7.3, and containing 10% defined newborn calf serum (NCS; Hyclone, Logan, UT) at 37^0 C in 5% CO_2-enriched air (29). All media and reagents will be tested for endotoxin to assure a concentration of <0.1 ng/ml. New cultures will be started monthly from frozen stocks.

8.2b *A549 Human Type II cell line: A549 Human alveolar Type II cells* (American Type Tissue Culture Collection, Rockville, MD; ATCC # CCL-185) will be maintained in Ham's F12K medium supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, adjusted to contain 90% 1.5 g/L sodium bicarbonate and 10% fetal bovine serum at 37^0 C in 5% CO_2-enriched air (29). All media and reagents will be tested for endotoxin to assure a concentration of <0.1 ng/ml. New cultures will be started monthly from frozen stocks.

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8.3 Bioassays

8.3a *Cytokine release assays*: Cells will be seeded in 96 well plates (1 x 10^5 cells per well) and allowed to attach for 2 hr. Fresh media supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, will be placed in each well and an aliquot of a $PM_{2.5}$ sample will be added to achieve concentrations ranging from 20 to 200 ug/ml. After 24 hr, media from each 96 well plate will be tested for cytokine release (A549 cells: IL-6, IL-8; RAW264.7 cell: TNF_{α}, and GM-CSF) using commercially available ELIZA kits (R&D Systems, Minneapolis, MN and Endogen, Cambridge, MA). Results from the cytokine release assays will be expressed as pg cytokine/ml media. Cytotoxicity of the $PM_{2.5}$ samples will be determined by measuring lactate dehydrogenase (LDH) release. In addition, viability of cells remaining in the wells will be determined by trypan blue exclusion or crystal violet staining. Samples showing significant toxicity to the cells (viability <90%) at the lowest concentration will be diluted and re-evaluated. Viability data will be used to determine the LD_{50} for each $PM_{2.5}$ sample and also as a means of normalizing the cytokine results.

The role endotoxin in the induction of cytokines will be determined in a subset of samples by running experiments with and without the endotoxin inhibitor, polymyxin B $PM_{2.5}$ samples will be tested for endotoxin using the *Limulus polyphemus* amebocyte assay (Whittaker Bioproducts, Walkersville, MD). The cytokine release assay will be run with and without polymyxin B on $PM_{2.5}$ samples with endotoxin concentrations greater than 0.1 ng/ml.

8.3b *Reactive Oxygen Species (ROS) assays: C*ells will be seeded in 96 well plates (1 x 10^5 cells per well) and allowed to attach for 2 hr. Fresh media supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, will be placed in each well and the cells preincubated for 3 hr with bacterial lipopolysacchride (LPS, E. coli serotype 0127:B8) at at concentration of 10 ng/ml (Imrich et al., 1999). Cells will then be washed, preincubated with 100 ul of 10 uM DCF-DA (dichlorodihydrofluorescein diacetate) for 30 min., washed with serum-free RPMI media and exposed to $PM_{2.5}$ at concentrations ranging from 20 to 200 ug/ml. Fluorescent intensities will be measured at an excitation wavelength of 485 nm and an emission wavelenth of 535 nm. Fluorescent intensities of cells treated with $PM_{2.5}$ will be normalized to the fluorescent intensities of cells treated with PMA (phorbol myristate acetate). (Chen et al., 1999).

8.4. Quality control

To standardize assays run on different days, each run will include standard urban particulate matter SRM 1649 (NIST, Gaithersburg, MD) as a positive control and titanium dioxide as a negative control.