Investigation of Cell Culture Media Infected with Viruses by Pyrolysis Mass Spectrometry: Implications for Bioaerosol Detection

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Mass spectrometry coupled with a pyrolysis inlet system was used to investigate media from cell cultures infected with viruses. Cell culture media is an intricate mixture of numerous chemical constituents and cells that collectively produce complicated mass spectra. Cholesterol and free fatty acids were identified and attributed to lipid sources in the media (blood serum supplement and plasma membranes of host cells). These lipid moieties could be utilized as signature markers for rapidly detecting the cell culture media. Viruses are intracellular parasites and are dependent upon host cells in order to exist. Therefore, it is highly probable that significant quantities of media needed to grow and maintain viable host cells would be present if a viral agent were disseminated as an aerosol into the environment. Cholesterol was also detected from a purified virus sample, further substantiating its use as a target compound for detection. Implications of this research for detection of viral bioaerosols, using a field-portable pyrolysis mass spectrometer, is described. (*J Am Soc Mass Spectrom* 1999, 10, 502–511) © 1999 American Society for Mass Spectrometry

The development of biological weapons by rogue nations and terrorist organizations poses a serious threat to the present and future welfare of global societies [1, 2]. A pressing need facing the scientific community is the advancement of technologies to combat these weapons of mass destruction. A particular necessity is the development of analytical devices capable of rapidly detecting and/or identifying pathogenic entities deliberately dispersed into the environment. Toward this goal, an array of instrumental and chemical systems [3, 4] have been designed. One of the more recent approaches uses a field-portable pyrolysis-mass spectrometer built by the U.S. Army [5]. This instrument samples air directly from the atmosphere using a virtual impactor to collect aerosolized substances onto a quartz frit located inside a microtube pyrolyzer.

Pyrolysis was chosen as the sample inlet for the field mass spectrometer because of its small sample requirements, high sample throughput, low power requirements, and simple design. The pyrolysis device vaporizes stable molecules intact, whereas nonvolatile and/or thermally labile molecules decompose into smaller molecular fragments [6, 7]. Analysis of complex

high molecular weight biomaterials, using pyrolysis-mass spectrometry (Py-MS), results in mass spectral peaks that can be attributed to specific biochemical precursors known as biomarkers.

Ongoing research in our laboratory, using Py-MS, is devoted to building a database of biomarkers to be used in the rapid detection of a multitude of bacteria, viruses, and toxins [8]. Mass spectrometry coupled with pyrolysis is a well established method for the elucidation of biomarkers [9–12] and the differentiation of whole cell bacteria [5, 13] and fungi [14, 15]. Viruses, however, have not been extensively studied by Py-MS. To our knowledge, only two reports describing the application of Py-MS to the study and characterization of viruses have been published. Tas and co-workers, in one of these reports, evaluated the use of Py-MS for differentiating cell culture media infected with viruses from blank media not infected with a virus [16]. In the other report, Windig et al. utilized Py-MS for testing poliovirus suspensions separated by column chromatography for DEAE-sephadex impurity [17]. The focus of both papers was on the use of pattern recognition techniques for differentiating pyrolysis mass spectra, but neither paper identified mass spectral peaks of taxonomic significance to either the virus or the cell culture media. A more thorough investigation of media containing viruses is therefore required in order to evaluate the

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potential use of Py-MS for detecting aerosolized viral media.

Viruses are an attractive choice for biological weaponry because of the debilitating consequences a viral infection could have on civilians and military personnel [18]. Several groups of viruses that are transmissible in aerosolized form are suspected agents for biological weaponry [18, 19]. These viral agents might not be purified prior to use, therefore detection schemes must account for situations in which only a small amount of the virus is present in a large quantity of culture media. The media in these samples would likely overwhelm any signal generated by the viruses and thus make direct detection of the viruses extremely difficult, if not impossible. On the other hand, the culture media itself should be amenable to detection. Detection of aerosolized culture media in the environment would strongly suggest the presence of a viral agent.

The objective of this study was to find unique biomarkers that eventually could be used to rapidly detect aerosolized culture media contaminated with viruses. For this purpose, a laboratory based Py-MS was used to investigate cell culture media infected with viruses. An advantage of employing mass spectrometry, in lieu of other virus detection methods such as serological techniques and the polymerase chain reaction, is shorter analysis times. The field-portable, direct-sampling Py-MS mentioned above is designed to automatically collect, pyrolyze, and mass analyze air samples within minutes. Mass spectrometry has the added advantage of being able to detect a wide range of different biological and/or chemical agents (not limited to a reaction with a specific antibody or primer).

All viruses pathogenic to humans require eukaryotic host cells for growth and replication. The most common eukaryotic systems employed are monolayer cell cultures and chicken egg embryos. The following report describes the investigation of these two media using Py-MS.

Experimental

Instrumentation

Mass spectra were collected on a triple quadrupole mass spectrometer (model ELQ 420, Extrel, Pittsburgh PA) modified with a Curie-point pyrolysis inlet. Power was supplied to the Curie-point coil using a Fischer Model 0310 (1 kW) rf generator. The basic design of this instrument has been described elsewhere [20]. Aliquots of 10 μ L of culture media supernatants and standard reagents were evenly coated onto Ni (358 °C) Curie-point wires and evaporated to dryness by rotation. The wires were held in quartz sample tubes and inserted into the mass spectrometer via a direct probe inlet. The temperature rise time of Ni wires is on the order of 210 ms. The equilibrium temperature of 358 °C was held for 9.9 s. The ion source was operated in the positive electron ionization (EI) mode with an energy of 70 eV.

The ion source and transfer line (quartz tube 5 cm long and 1.2 mm i.d.) temperatures were maintained at 210 °C. Full-scan spectra were collected for the mass spectral scan range extending from m/z 210 to m/z 420 (unless stated otherwise) with a scan rate 3.6 scans/s.

Data Analysis

Mass spectra for data analysis were acquired by averaging 10 scans taken from the total pyrogram (total ion current). Principal component analysis (PCA) was carried out using the RESOLVE software package [20]. Mass spectra were collected as a set of raw intensities over the full scan range. The data were normalized to total intensity to account for differences in sample sizes and variances in total ion current. In addition to normalization, each mass spectrum was mean centered by subtraction of the average mass spectrum prior to PCA.

Sample Preparation

All cell culture reagents and standard chemicals were obtained from Sigma Chemical (St. Louis, MO) and used without further purification. Feline kidney cells (CRFK) infected with feline enteric coronavirus (FECV) and CRFK cells not infected were cultured at the Armed Forces Institute of Pathology (AFIP) in Washington D.C. The feline kidney cells were purchased from American Type Culture Collection (ATCC 94CLL Batch F-14398) and were grown *in vitro* using growth and maintenance media. The complete growth media consisted of: Eagle's minimum essential media (EMEM) supplemented with 10% horse serum, 1 mL of L-glutamine per 100 mL of media, and 1 mL of antibiotic-antimycotic (100 $\times$) per 100 mL of media. The antibiotic-antimycotic solution contained 10,000 units of Penicillin, 10 mg of streptomycin, and 25 μ g of amphotericin B per mL of 0.9% NaCl solution. Cell culture supernatant was prepared by first detaching the cells from the culture dishes using trypsin-EDTA, followed by a rapid freeze-thaw step to lyse the cells releasing intracellular constituents and the viruses. Finally, the supernatants were prepared by slow speed centrifugation (9000 G) to remove large cellular components. Cell lysis and subsequent supernatant preparation was initiated after the cell cultures were observed to be at full cytopathic effect (CPE).

Vero (African green monkey kidney cells; ATCC ECL-81 Batch F-11497) cells and Vero cells infected with Venezuelan Equine Encephalitis (VEE) were also prepared at the AFIP. The complete growth media used to culture the cells consisted of EMEM supplemented with 5% fetal bovine serum, 1% antibiotic-antimycotic (penicillin 10,000 units, streptomycin 0.1 mg, and amphotericin B 0.25 μ g per mL of 0.9 NaCl), and 0.292 g of L-glutamine per liter of growth media. Supernatant from the cultures were collected using a procedure similar to the one described above for the CRFK cultures. VEE preparations were γ killed (irradiated) prior

to use in a Biosafety-Level 2 laboratory. The supernatant preparations were analyzed without further pretreatment.

Intact Vero cells analyzed in this study were prepared as described above except that the cells were not subjected to lysis. The cells were isolated from the growth and maintenance media by centrifugation at 9000 G for 10 min in 0.22 μ m acetate centrifuge filters (Micron Separations; Westboro, MA). The isolated cells were washed twice with water and resuspended in 0.9% NaCl before analysis by Py-MS.

Allantoic fluid from a chicken embryo (9–10 days old) infected with influenza A virus (incubation period 48 h) was ordered from American Type Culture Collection (ATCC VR-99; strain: A/Swine/1976/31; original source: hog in Iowa, 1931) and analyzed without further pretreatment.

Purified mouse hepatitis virus (MHV) samples were prepared at the University of Colorado Health and Science Center. The cell line used to propagate MHV were 17cl.1 cells, which is a spontaneously transformed BALB-c mouse fibroblast cell line. 17cl.1 cells are grown in Dulbecco modified Eagles medium supplemented with 10% fetal bovine serum and 2% antibiotic solution containing penicillin, streptomycin, and fungizone. The virus MHV-A59 was added at a multiplicity of infection (MOI) of 0.1, incubated at 37 °C and 5% CO₂ for 1 h, the virus inoculum was removed and fresh media at pH 6.0 was added. The supernatant was harvested at 24 h post infection. Cell debris was removed by centrifugation (1000 rpm for 5 min). Supernatant was quickly frozen in dry ice/EtOH bath and stored at –80 °C. The virus was purified on a sucrose gradient. The viral pellet was resuspended in tris malate buffer at 1 mg per mL and examined without further purification.

Results and Discussion

The mass spectra produced from pyrolysis of media from cell cultures are inherently complicated because of the many different chemical constituents required to grow and maintain viable cells in vitro. Growth and maintenance media are isotonic solutions that furnish cells with nutrients and vitamins while regulating the pH and electrolyte balance [21]. The growth media is also routinely supplemented with blood serum (5%–20% v/v) which provides the cells with additional nutrients and growth factors such as hormones, trace elements, minerals, and lipids [21]. Antibiotics are also added to the culture to inhibit bacterial contamination. Viruses are usually harvested by physical lysis of the infected host cells, a practice which further complicates the culture milieu by releasing intracellular biochemical constituents.

Figure 1a, b displays the mass spectra of supernatants recovered from a Vero cell culture infected with VEE and media from a Vero cell culture that was not infected. Many of the peaks observed in the two spectra are fragment ions generally attributed to the pyrolysis

of proteins and free amino acids [22, 23]. In addition, ion fragments from the other classes of biomolecules (lipids, nucleic acids, and carbohydrates) also contribute to the spectral peaks observed for the cell culture media [24]. The two mass spectra are similar both qualitatively and quantitatively. The relative proportions of biomolecules apparently remains constant between the two cell cultures despite one being infected with the VEE virus.

VEE is composed of a strand of nucleic acid encapsulated in a protein shell which is covered by a lipid bilayer. Biomolecules, regardless of their origin, are thermally degraded to the same degree. Therefore, pyrolysis of the virus is expected to produce molecular fragments of similar nominal masses as produced by the culture medium. Because the two mass spectra in Figure 1a, b are nearly identical it is presumed that the contribution of the virus is negligible, otherwise a noticeable difference in peak ratios between the two spectra would be visibly apparent. This result was not unexpected, considering that the yield of viral mass from infected cells is small (~0.1% w/w) relative to the overall masses of the host cells and growth media. The chemical signal originating from the cell culture media dominates the spectra of the unpurified viral media overwhelming the peaks generated from the viruses. This result is consistent with the previous study by Tas et al. [16] which concluded that purification appeared to be a prerequisite for reliable classification of the virus.

Subsequently, the cell culture media was examined in greater detail because a biomarker distinct to the virus could not be easily detected from the matrix. The planned course of this analysis was to systematically determine which of the constituents of the cell culture media contributes the greatest to the overall mass spectra generated by the supernatants. The constituents for both the infected cell culture media, and supernatants from these media are listed in Table 1. The constituent recognized as having the strongest influence would then be thoroughly examined for potential biomarkers that would facilitate rapid detection of media commonly associated with viruses.

The mass spectrum from a complete growth medium consisting of EMEM supplemented with 5% fetal bovine serum and 1% of a 100 $\times$ diluted antibiotic-antimycotic solution is shown in Figure 1c. Visibly, this mass spectrum is comparable to the spectra from cell culture supernatants (Figure 1a, b). The most noticeable difference being the higher intensities of m/z 70 and 84 in the complete growth medium spectrum. The varying intensities observed between the spectra in Figure 3 are most likely the direct result of the metabolic activity of the cultured cells. Aside from the variability in peak intensities, it appears that the complete growth media is a dominant contributor to the overall distribution of mass spectral peaks.

Consequently, each constituent (EMEM, fetal bovine serum, and antibiotic-antimycotic solution) of the complete growth media was examined. Due to the

Figure 1. (a) Vero cell culture infected with VEE, (b) Vero cell culture not infected with VEE, and (c) complete growth media. Note how closely the two cell culture media resemble the complete growth media.

complexity of the individual spectra, principal component analysis (PCA) was used to help interpret the data. The score plot of the first two principal components (59% of total variance) obtained for the mass range beginning with m/z 210 and ending with m/z 420 is shown in Figure 2. Each datapoint, on the score plot,

represents a mass spectrum from either the complete growth media or one of its constituents. The similarity between two mass spectra is measured by the distance between their respective datapoints on the score plot [25]. The closer the distance between two datapoints, the more these two mass spectra resemble one another.

Table 1. Components of cell culture media and cell culture supernatant

Infected cell culture medium:
• Monolayer cells used to propagate viruses
• Viruses
• Complete growth media
■ Growth and maintenance media (EMEM)
■ Blood serum supplement
■ Antibiotic-antimycotic
Supernatant from infected cell culture media:
• Cellular fragments from lysed cells
• Viruses
• Complete growth media

Datapoints representing the fetal bovine serum spectra clustered nearest to the respective points for the complete growth media. Therefore, the mass spectral peaks observed for the complete growth medium originate mainly from the blood serum constituent. The influence of the serum was determined to be the greatest for the mass range of m/z 210 to m/z 420.

Blood sera was further analyzed to determine if characteristic biochemical molecules, in the mass range extending from m/z 210 to m/z 420, could be identified. The mass spectra of horse and fetal bovine serum are shown in Figure 3a, b. These two sera are commonly utilized in mammalian cell cultures. Both spectra exhibit ions that correspond to cholesterol and free fatty acids (FFAs). Distinguishing cholesterol ions include its molecular ion at m/z 386 and fragments ions at m/z 368, 353, 326, 301, 274, and 213. Ions of FFAs identified in the horse serum spectrum included: stearic (284), linoleic (280), and palmitic (256) acid. In addition to the dehydration peak $[M^{+}-18]$ of oleic acid (264), stearic and palmitic acids were also observed in the fetal bovine serum mass spectrum. The mass to charge ratio values for the FFAs detected are given in Table 2. The appearance of an intense linoleic peak in the horse serum

spectrum and its almost complete absence in the spectrum of the fetal bovine serum agrees with reported concentrations of this acid in the two blood sera [26]. The total concentration of FFAs in horse serum and fetal bovine serum are 0.49 and 0.35 $\mu\text{mol/mL}$, respectively [26]. The identity of each lipid moiety was verified by running pure standards, product ion scans of the molecular ions, and comparisons with the NIST database.

The relative intensities of the cholesterol and FFA ions were enhanced by using 358 °C Curie-point wires preferentially to higher temperature wires. The lower pyrolysis temperature helped reduce the contribution made by other components less volatile than the lipid compounds. Meuzelaar and Huff [27] reported the detection of cholesterol and long chain aliphatic fragments in human white blood cells using 358 °C Curie-point wires. Their spectra were generated by pyrolyzing the sample on the wire, removing the spent wire from the quartz sample tube, and then reheating the condensate that formed on the inside of the quartz sample tube. The spectra displayed in this report were all generated by direct pyrolysis from the Curie-point wire. This technique is simple in practice and unique because underivatized fatty acids are rarely employed for taxonomic studies [28].

Molecular ions for the FFAs were observed within the first 5 to 20 scans of the pyrogram (100 total scans). After this period, the absolute intensity of these ions decreased quickly to the point that they were no longer detectable. Evidently the initial rapid surge in temperature caused distillation of the intact FFAs, but as exposure time to the heat increased, so did thermal degradation of the fatty acids. Evidence of this effect was observed for the m/z 386 to m/z 368 peak ratios of cholesterol. In the first part of the pyrogram the relative intensity of m/z 386 exceeds m/z 368, but the opposite is true in the later part of the pyrogram.

The two major sources of lipids in cell culture media are lipoproteins carried in the blood serum and the plasma membranes of the host cells. The mass spectrum generated by a standard mixture of bovine plasma lipoproteins is shown in Figure 4a. Cholesterol and FFA ions can easily be identified in this mass spectrum. Vero cells were analyzed in order to determine if lipid ions could also be detected for eukaryotic host cells. The cells were isolated and washed in water to remove the complete growth media and then resuspended in saline solution prior to analysis. The mass spectrum of the isolated cells is shown in Figure 4b; peaks for cholesterol and the FFAs can be identified in this spectrum. The detection of the host cell lipids is important for cell lines that can be grown successfully on serum-free media. The use of such cultures would effectively eliminate the rich source of lipids carried in the blood serum.

The Vero cells analyzed in Figure 4b were grown and maintained on a medium supplemented with fetal bovine serum. Lipids from the blood serum are incorporated, without modification, and used directly by the

Figure 2. The complete growth and maintenance media is designated by C, EMEM by E, fetal bovine serum by F, and the antibiotic-antimycotic solution by A. The fetal bovine serum and antibiotic-antimycotic solution were diluted using deionized water to equivalent concentrations found in the complete media.

Figure 3. (a) Horse serum and (b) fetal bovine serum. The blood sera were diluted 10 fold with deionized water to match their concentrations used for cell cultures.

cells so that the respective lipid profiles are expected to be comparable [29]. The mass spectra of fetal bovine serum (Figure 3b) and the isolated Vero cells do show similar relative intensities for the cholesterol and FFA ions.

Media collected from cell cultures used to propagate

viruses were reexamined for lipid biomarkers in the m/z 210 to m/z 420 mass range. The spectra of FECV and VEE infected cell culture media are shown in Figure 5a, b. The supernatant, recovered from these two cell cultures, represent crude viral preparations anticipated to be encountered in field scenarios. Both media show very intense mass spectral peaks corresponding to cholesterol and free fatty acids. FECV was cultured in a growth medium supplemented with horse serum. As expected, the mass spectrum from the FECV infected medium is largely reflective of the horse serum (Figure 3a). Likewise, the mass spectra of the VEE medium (supplemented with fetal bovine serum) and fetal bovine serum (Figure 3b) are comparable.

Table 2. Molecular and fragment ions of free fatty acids

Fatty acid	Molecular ion	Fragment ions
Palmitic acid	256	227 & 213
Stearic acid	284	255, 241, 227, 222, & 213
Oleic acid	282	264, 235, & 221
Linoleic acid	280	262, 223, & 210

Figure 4. (a) Lipoprotein standard from bovine plasma (predominately high density) and (b) isolated Vero cells free of complete growth media. Characteristic ions of cholesterol and fatty acids are observed in both spectra.

Chicken egg embryos are sometimes utilized to propagate viral strains not easily grown in cell cultures. The egg itself is injected with the virus and incubated for a specified amount of time required to grow the virus. The mass spectrum of allantoic fluid recovered from a chicken egg infected with Influenza A virus is presented in Figure 6a. The ions of cholesterol and the FFAs are clearly visible in the spectrum of the allantoic medium.

Because of the ubiquitous presence of cholesterol and FFAs in animal cell cultures, their presence in an aerosol sample could provide a fast and simple method for detecting media commonly used to propagate viruses. Cholesterol, in particular, is an excellent candi-

date biomarker for the detection of cell culture media by Py-MS because it produces intense molecular and fragment ions. The corresponding mass spectral peaks of cholesterol are easily discernable and provide a rapid scheme for differentiating eukaryotic cell cultures from prokaryotic culture media. Cholesterol is not generally found in media used to cultivate bacteria or in the bacteria themselves. Figure 6b shows the spectrum of *Yersinia pestis* grown in trypticase soy broth. No cholesterol ions are observed in this mass spectrum.

Purified viral preparations may also be encountered in the field. This issue was briefly addressed by examining purified mouse hepatitis viruses; the spectrum is displayed in Figure 6c. Peaks corresponding to chole-

Figure 5. (a) Cell culture supernatant infected with FECV and (b) cell culture supernatant infected with VEE.

terol are present in the MHV mass spectrum. Cholesterol is known to be incorporated into the lipid membranes of MHV during intracellular virus assembly [30]. The cholesterol biomarker can thus be used to detect the presence of both purified and nonpurified viruses.

Conclusion

Results from this study represent a preliminary investigation of cell culture media infected with viruses using a pyrolysis-triple quadrupole mass spectrometer. This study showed that cholesterol and FFA biomarkers could be identified from the mass spectra generated by Py-MS of cell culture media commonly used to proliferate viruses.

We believe that these biomarkers will allow detection of aerosolized viral media in real time using a field-portable, ion trap Py-MS. To substantiate this proposal, an extensive examination of aerosolized viral media using the field-portable unit will need to be conducted in the near future. As part of this study, limits of detection and potential interferents such as diesel fuels, cleaning reagents, pesticides, automobile exhausts, and other common background constituents will also need to be evaluated. The concentration of aerosolized cholesterol is not expected to be high for normal atmospheric samples, thus validating its use as a targeted marker for detection of culture media.

This rapid approach (detection of cholesterol and

Figure 6. (a) Allantoic fluid (chicken egg embryo) infected with Influenza A virus, (b) *Y. pestis* grown in trypticase soy broth, and (c) purified MHV.

FFA biomarkers within minutes) is limited to a pre-warning/first alert detection followed by more specific viral identification schemes. In situations involving the expected release of bioaerosols, atmospheric samples would continually be monitored for any substantial

increase in cholesterol and FFAs. Real-time detection of these biomarkers, above normal background levels, would give personnel in the vicinity of an early warning of the potential release of the pathogenic agent. Py-MS provides a rapid/nonspecific way to detect

these viral biomarkers without lengthy sample preparation steps and complex ionization/mass spectrometry instrumentation.

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