Mass Spectrometry of Breath for the Diagnosis of Infection and Exposure

Joany Jackman and Owen Moss

The inability to distinguish among people infected by agents of biological warfare, those infected by more benign agents, and those who simply perceive relevant signs and symptoms has been a major impediment to the rapid resolution of biological warfare attacks. The initial signs and symptoms reported for infection with biological warfare agents mimic those of more common illnesses such as the flu; therefore, an attack of this ilk could go unrecognized initially, wasting valuable resources and causing major health and economic impacts. One means to reduce or alleviate the threat posed by the use of biological warfare agents would be to quickly determine the extent of actual infection. Here we describe our approach to developing a rapid, sensitive, and reliable method to diagnose pulmonary infection presymptomatically using mass spectrometry.

INTRODUCTION

A critical need for the effective protection of troops and civilians against biological weapons of mass destruction (WMD) is the ability to rapidly detect infection. The problem is that signs and symptoms of exposure to biological WMD are quite general, are somewhat subjective, and can easily be confused with more common and less serious maladies. In general, detection of infection occurs when physical signs are observed and symptoms are reported by exposed people. Following an announced terrorist attack with an aerosolized biological agent, medical resources would be stretched significantly as evaluation and treatment of larger numbers of the population became necessary. Those affected would include people who are exposed and subsequently infected, people who are exposed but fail to inhale sufficient organisms to become productively infected, and people who are both unexposed and uninfected yet seek medical attention. This last category is often referred to as the "worried well."

Remediation of such an event via similar treatment of actual infected and uninfected populations is problematic for several reasons. By treating both populations equally, valuable medical resources are wasted on the uninfected. If, for example, a hoax incident were to occur, the misdirected efforts of hospital and emergency personnel to triage and evaluate uninfected civilian populations could endanger the lives of people with real medical emergencies and traumatic injuries. In addition, the supply of appropriate vaccines or antibiotics for civilian populations around the sites of an attack, both urban and suburban, may be severely limited or nonexistent and create logistics burdens for military units.

In the case of a biological warfare or bioterrorist attack on a naïve civilian or military population, remediation through broad-spectrum treatment of the exposed population with antibiotics or prophylactic vaccination is not without risk. The profile of the average civilian population, unlike that of an active military unit, is apt to include children, pregnant women, the chronically ill, and the aged. The efficacy and safety of current vaccines are unknown for many of these groups. Antibiotics themselves are not without side effects.

There is a direct and urgent need for a means to rapidly triage affected populations for signs of infection as soon after an incident as possible. We have developed a sensitive diagnostic device to meet this challenge. Early diagnosis would be accomplished by detecting biological molecules produced by the host in response to infection. In this model, humans are the ultimate and direct sensors. We are developing a method to indirectly detect the infectious organism and directly detect the infectious process. If the positively infected population could be sorted from the uninfected population quickly and early in the scenario, many of the risks of a biological warfare or bioterrorist attack could be reduced or neutralized by direct intervention at the point of attack or the point of care, and limited resources could be preserved for the most seriously ill.

INFECTION

Host Immune Response

Following exposure to bacteria, a foreign protein, or a virus, a number of events occur within the host to mount an immune response against the invading material. These events are initiated in the tissue where microbial invasion occurs. By necessity, a localized immune response is immediate. Immune proteins stored within cells are secreted. Some proteins are secreted at low levels, are rapidly taken up by surrounding cells, and are therefore unavailable for collection. However, some immune proteins are secreted into the external spaces of the lung in high concentrations. Once secreted, they diffuse slowly away from the site of infection, forming a concentration gradient by which immune cells distant to the site of infection can sense and follow to that site.

Like PCR (polymerase chain reaction), which amplifies the genetic material rapidly, the molecular events that trigger the host immune response amplify the signal resulting from an infection exponentially. Activation of the host immune system results in the release of a cascade of molecules that sequentially activate additional molecular responses. The molecular events involved in immune system activation are appropriately robust, are focused at the site of the infection, and *continue through all phases of infection*, including remediation. This last point has significance for the potential value of monitoring host response to detect infection: while current treatment modalities are devised based on the *predicted* responses of an entire population, monitoring in near real time would make it possible to assess the effectiveness of treatment as well as any adverse effects of the treatment in the host.

The host cell response to infection in the lung has been well documented. However, this information is primarily limited to the investigation of bacterial pneumonias and influenza.[1–3] The candidates, *Bacillus anthracis, Yersinia pestis*, Venezuelan equine encephalitis, and *Staphylococcus* enterotoxins, are all effective weapons when delivered by an aerosol route. Therefore, since the initial host cell response to infection is local, it should be possible to detect the earliest signs of infection in the exhaled products of the lung.

Candidate Biomarkers

For our approach to be useful in detecting early signs of infection, candidate biomarkers must meet the following criteria:

- They must be secreted immediately (<2 h).
- Their production should be robust to provide the necessary amplification of signal required to detect low numbers of agents.
- Their production or stability must be relatively long-lived (detectable for hours or days) so that they can be assessed over a wide time frame following exposure.
- They should be easily detectable, since complex sample processing would severely slow down and perhaps confound the analysis.
- Their signal should be applicable to the normal population as a whole following infection with biological agents.

Although this is a tall list of requirements, the secreted proteins produced by immune cells following infection appear to satisfy all these criteria. Known collectively as "cytokines," there are currently more than 200 known proteins secreted in response to foreign body invasion, and new candidates are added yearly.[4,5] Arachidonic acid metabolites, which include prostaglandins, eicosanoids, and lipoxins, are also known to be produced in large amounts and in various structural isoforms in response to immune stress[6] (Fig. 1). This category of signal molecules includes more than 100 different reactive lipids that are structurally derived from arachidonic acid and have been implicated in host responses to infection. Both these protein and lipid markers (and others as yet undiscovered) would presumably be good indicators of infection if they could be measured directly and rapidly.

Cytokines are similar to hormones in that they are small, secreted proteins with modulatory effects on other cell types but dissimilar to hormones in that these effects are exhibited locally (paracrine and autocrine)

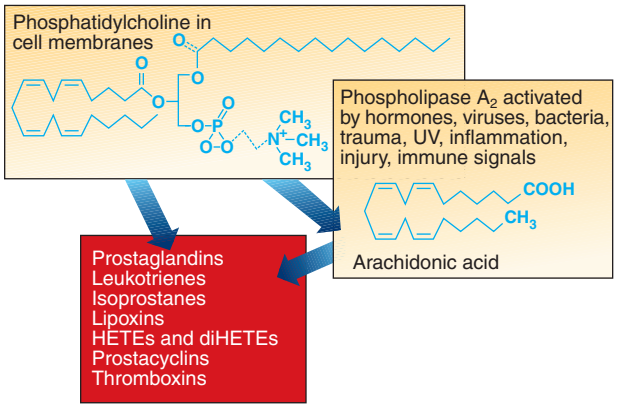

Figure 1. The synthesis of arachidonic acid metabolites is an important factor in early host immune responses. Arachidonic acid provides the foundation for the synthesis of more than 10 different prostaglandins through the multiple enzyme systems, more than 40 hydroxyeicosatetraenoic acids (HETEs) and substituted HETEs through the p450 and lipoxygenase systems, and more than 20 leutrotriene variants. These products are formed while in intact lipids or after release by phospholipase A_2. All of these molecules are involved in host responses to infection and stress.

rather than at a distance (endocrine) (Table 1). However, not all cytokines are expressed in each tissue type, suggesting that there may be some spatially restrictive pattern to the expression of cytokines based on the site of infection.[4] The use of cytokines in our approach is further discussed later in this article.

Susceptibility

Age, stress, genetics, sensitivity to drugs, and underlying disease status all influence a person's ability to fight infection. Therefore, even similarly exposed people may not demonstrate equal infectivity to a particular agent. For example, if a 58-year-old diabetic female became productively infected, it would not necessary mean that a 29-year-old healthy male located in the same area would also become infected. The advantage of our approach is that it would capitalize on *individual* rather than population approaches to treatment. A rapid system for detection of disease-specific markers would prevent inappropriate use of resources and unnecessary risk.

SAMPLING

Ultimately it would be most beneficial to detect signs of infection *before* patients exhibit symptoms. Any device used for this purpose should be portable and amenable to rapid sample analysis as a point-of-care system. Samples should be easy to obtain, i.e., with little stress on the patient, should present a low logistics burden on the medical staff, and should be easy to process.

In our case, we chose to collect water vapor and particle droplets of exhaled breath as a sampling system (Fig. 2) to monitor active infection in the lungs and waste products from infection expelled through the lungs. Others have used volatiles such as nitric oxide levels in breath to monitor infection. Although nitric oxide has proven to be an excellent marker, nitric oxide basal levels have been shown to vary considerably from person to person.

Anyone who has spent time outside on a cold day and could "see his or her breath" has visualized the process by which exhaled breath can be captured. An active, healthy male may exhale as much as a quart of water per day. Entrained within this water vapor we expected to find larger mass molecules such as lipids, proteins, and even whole viruses. The protein concentration of breath is highly variable, depending on the collection method, and has been measured by us and in a number of other studies looking at exhaled breath condensates (EBC). The collector design is discussed in more detail below.

As illustrated in Fig. 2, we proposed to use mass spectrometric patterns of EBC to determine infectious status, most importantly because breath fits the criteria mentioned previously (easily obtained, low logistics burden, and low patient stress). We chose host response molecules, primary cytokines and arachidonic acid metabolites (Fig. 1)[1,6] for several reasons. For example, while several thousand *Bacillus anthracis* spores may be necessary to induce infection, less than 10 organisms can induce disease if a person is exposed to *Francisella tularensis*. In such cases, even where 100% recovery of free swimming organisms can be demonstrated in models or controls, it is likely impossible to efficiently recover microbes from a host immediately following a biological warfare attack. Once microbial invasion has taken place, host cells may

Table 1. Comparison of characteristics.

Cytokines	Hormones
Act locally	Act at a distance
Produced by many cell types (immune, epithelial, etc.)	Produced by specialized cells (thyroid, adrenal, etc.)
Synthesized transiently following activation	Synthesized constitutively following activation
Usually inactive in serum or plasma	Active in serum or plasma
Diffuse from tissues, slowly forming a gradient from the site of action	Easily measured in blood and can be correlated with physiological activity

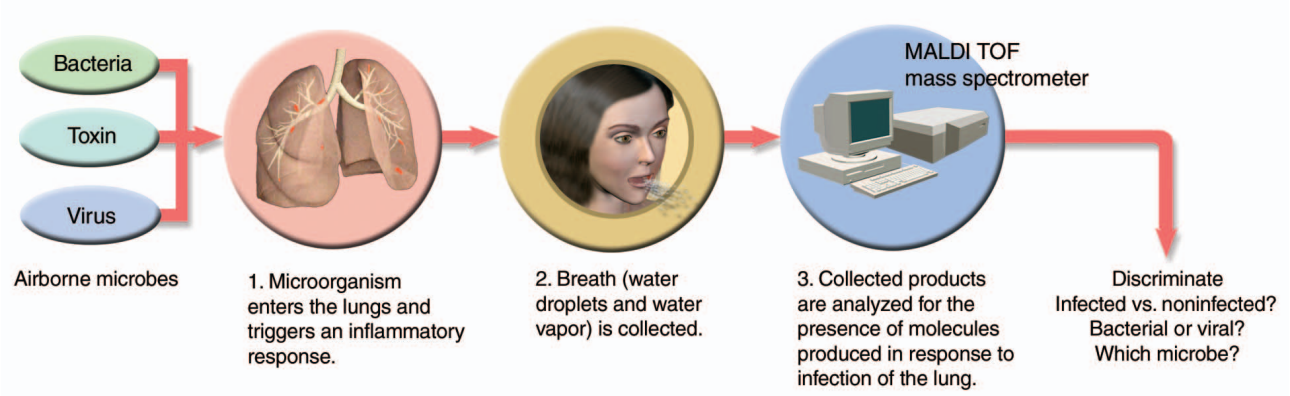

Figure 2. Conceptual schema for diagnosing infection from exhaled breath condensates using MALDI TOF mass spectrometry. It is anticipated that this system will be able to discriminate infected from uninfected people, should be able to distinguish classes of infection or exposure to toxins (bacterial, viral, and toxins) based on patterns, and might even provide information concerning the infectious agent (Gram negative, Gram positive, species information).

internalize the bacteria, virus, or toxins so that the agent cannot be recovered without lysis of the host cells. Successful detection at later times—when microbes have successfully proliferated throughout the host and appear extracellularly in blood—would be possible, although valuable treatment time may be lost.

PROTOTYPE DEVICE

The unique part of our prototype monitoring system is the use of Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI TOF) mass spectrometry (Fig. 3). In MALDI, samples are mixed with a solvent in which a compound known as a "matrix" is dissolved. Matrix refers to any one of a number of light-absorbing molecules that are also capable of transferring this adsorbed energy to nearby molecules. Interrogation of the sample mixed with the matrix results in the transfer of energy and subsequent ionization of the sample.

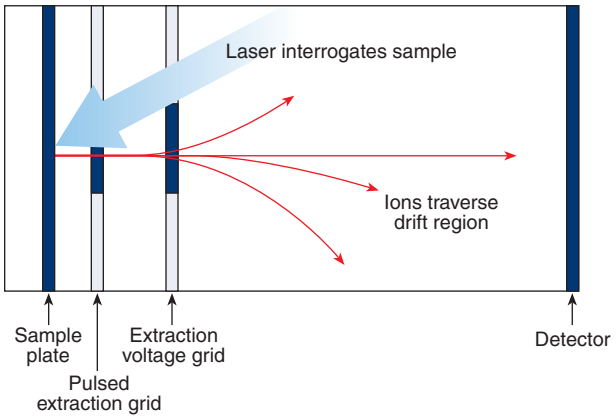

Figure 3. Schematic of a linear MALDI TOF mass spectrometer. The principle of MALDI TOF is to determine the mass of a molecule. Mass is determined by the time it takes for an ionized form of the molecule to travel in a field-free drift region from the source to the detector. Ions are produced when the sample analyte is mixed with an energy-absorbing matrix and the sample mixture is interrogated by a laser. This calibrated time measurement is recorded as the mass/charge ratio, where additional charges on a single molecule decrease the flight time of the ion in the drift tube proportionally to its mass. (Adapted from Ref. 7.)

MALDI TOF was selected as a candidate technology for breath detection for several reasons. First, it can be applied to molecules from a wide mass range (0–300 kDa) so that multiple markers can be visualized simultaneously. Second, MALDI is extremely sensitive—reported to measure yoctomole levels of peptides. However, it is relatively insensitive to contaminants such as detergents as compared to other mass spectrometry systems, thus reducing the need for extensive sample preparation. In addition, the MALDI process of ionization and detection is rapid (<1 s).

Initially, the prototype MALDI TOF mass spectrometer could be stationary, but the technology selected for this type of detector must ultimately be transportable and should be capable of miniaturization (<100 lb). Others at APL (see, e.g., the article by Antoine et al., this issue) are developing portable MALDI TOF mass spectrometers for use in field applications. A portable diagnostic device would allow evaluation of an exposed population on site for defined, early cellular responses to infection.

DETECTION OF CYTOKINES BY MALDI

Cytokines are functionally categorized as pro-inflammatory, anti-inflammatory, and chemotactic (also known as "chemokines").[5,8] A majority of cytokines have molecular weights below 30 kDa in their monomeric form and most chemokines have molecular weights in the ranges of 8 to 10 or 15 to 20 kDa (Table 2). This size range is extremely well suited to analysis by MALDI TOF mass spectrometry. Other technologies are available, However, with SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), the cytokines are nearly indistinguishable. SDS PAGE

Table 2. Characteristics of known cytokines involved in infection.

Cytokine[a]	Predicted MW[b] (kDa)	Observed MW[c] (kDa)	Glycosylation (N- or O-linked)	Immune cell source[d]
TNFα, β	26.0	17		T/M
IFNα	20.0	16–27	N	T/M
IFNβ	20.0	28–35	N	—
IFNγ	17.1	20, 25	N	T
IL1α, β	17.5, 17.3	17–20	N	M
IL-2	15.4	15–20	O	T
IL-4	15.0	15–19	N	T
IL-6	20.8	26	N	T/M
IL-8	11.1	6–8	—	M
MIP1α, β	8.6	8–20, 7.8	O	M/T
MIP2 (GROα) (GROβ)	11.4	7.9	—	M
MCP1, 2, 3	8.7	8–18	O	M
RANTES	8.0	8	O	T
GMCSF	11.6	22	N	T/M

[a]GMCSF = granulocyte/macrophage colony stimulating factor, IFN = interferon, IL = interleukin, MIP = macrophage inhibitory protein, TNF = tumor necrosis factor.
[b]Data compiled from Refs. 4 and 5. Molecular weight (MW) is based on reported relative MW from murine sources.
[c]Data compiled from Ref. 9.
[d]T = T cell-derived, M = macrophage-derived.

separates proteins based on their relative molecular mobility and is commonly used to identify proteins based on both their antigenic determinants and mass. In general, ELISA (enzyme linked immunosorbent assay) is used to antigenically detect and distinguish cytokines[10] and, in conjunction with Western blotting, is commonly used to quantify cytokine responses. Although these methods have been refined and have proven to be among the most accurate, specific, and sensitive technologies validated to date, ELISA lacks the immediate detection capabilities required for rapid analysis of large numbers of victims following an attack. However, mass spectrometry, because of its high resolving power for proteins of low molecular weight and its rapid sample analysis time, is a good candidate technology for the specific analysis of cytokines.

As part of the development of the mass spectrometry methods for sample analysis, we examined purified cytokines from a variety of species for their ability to ionize by MALDI. Several matrices and solvent conditions were compared among all the cytokines. Finally, a set of conditions was optimized to permit ionization of cytokine sets. These cytokine mixtures were grouped based on the likelihood that the molecules were induced, at times simultaneously, at early time points.

As shown in Fig. 4, cytokines IL-1, IL-6, and TNFα were all detectable as mixtures and when admixed in a 1:1 ratio with sputum. Cytokines were detected in mixtures at nanomole levels, although the intensity of the signal for each was not equivalent. The ratio used represents an unrealistic amount of sputum to be found in breath samples (as discussed in greater detail below). It is significant that even at these levels, sputum did not interfere with the detection of cytokines. This indicates that, unlike PAGE, MALDI can resolve proteins with masses that differ by less than 500 Da. In addition, detection of all three cytokines was accomplished in the presence of a 24 molar excess of bovine serum albumin. This is significant, as serum albumin is known to leak from the vascular spaces into the lung and is a major protein component of lung fluid.

Although forced sputum as used in these samples does not represent fluid derived from the lung, it does represent a material that might also enter the sampler when EBC is collected. Therefore, it is notable that neither sputum- nor respiratory-associated proteins such as serum albumin interfered with the detection and resolution of the cytokines in a neat (unprocessed) sample.

DETECTION OF CYTOKINES IN EXHALED BREATH CONDENSATES

Design of the Collection System

To conclude these studies, we first needed to be able to collect EBC with high efficiency. We evaluated several collector systems to be used in mouse and piglet model studies. For mice, exhaled breath was collected with a Cannon nose-only exposure tower (Lab Products,

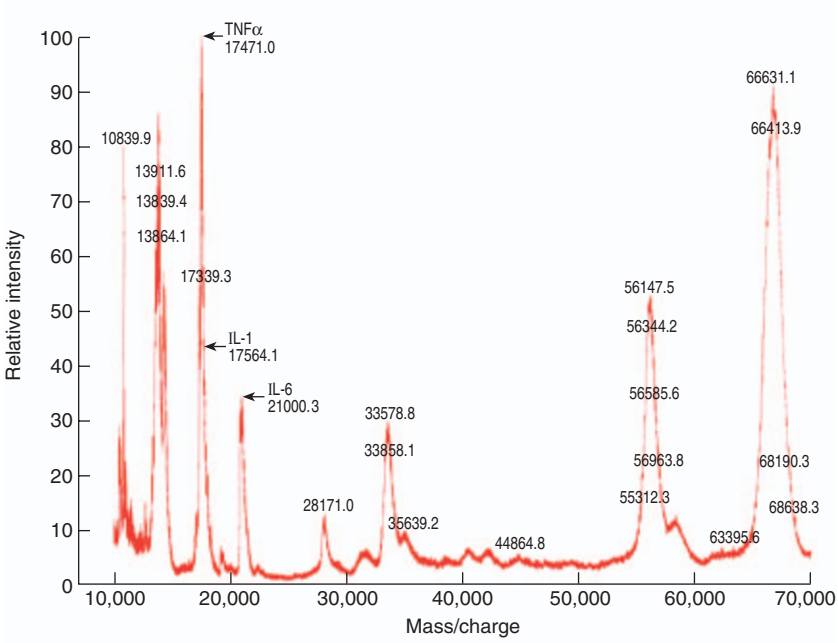

Figure 4. Resolution of cytokine mixtures in respiratory-like backgrounds. Three commercial cytokine preparations (IL-1, IL-6, and TNFα) were mixed at equal molar amounts (nmol). The *x* axis is mass in daltons over charge (+1, +2, etc.) and the *y* axis represents the relative intensity of the peak. Bovine serum albumin (BSA) was present in the samples as a stabilization agent at 24 molar excess over the concentration of individual cytokines. Cytokine mixtures were then admixed with forced sputum collected from a healthy volunteer. Peaks at 66 and 33 kDa result from the singly and doubly charged species of BSA.

Seaford, DE) modified to create a virtual mask for each rodent. The system allowed simultaneous sampling of breath from up to 50 mice. A cold trap was attached to the sampling system to collect water vapor and protein.

Initial calibration and testing of the system were done with water vapor, and 13-nm-dia. carbon black particles were then used to model the potential loss of exhaled protein clusters in this collection system. We hypothesized that particles of this size might be in the appropriate range of coalescing protein clusters as they become independently airborne or as they would exist inside the secondary liquid droplets that form when a soap-like film breaks. The selection of the 13-nm-dia. carbon particle size as a starting point was based on the observation that, following exposure to radon gas, radon daughters are exhaled. Inhaled radon decays to polonium, bismuth, and lead, which are solids at room temperature. These single ionized atoms attach to the nearest airway surface, particle, or cluster of molecules. If the particles are in the tens to hundreds of nanometer-diameter size range or rapidly grow to that size range, the radon daughters are exhaled and the absorbed dose decreased.[11,12]

The major source of breath protein can only be speculated at this time. Except during violent air movements in the upper airways, the generation of aerosol from fluid lining the conducting airways is not expected to be significant. It is possible that aerosol is generated by yet-to-be-demonstrated means in the deep alveolar spaces. This protein aerosol may be produced by the movement of air through the pores of Kohn,[13] by airway reopening,[14] or possibly by the dynamics of an alveolar surface network of bubbles.[15]

As a worst-case scenario, the smallest stable aerosol that we could generate was chosen to test the penetration of breath protein through the exhaled breath sampling system. If a 13-nm-dia. aerosol could penetrate from the location of the nose of the animal, through the sampler, to the collector (cold trap), then any larger clusters would also penetrate as long as they remained below the larger particle sizes, where impaction and sedimentation dominate particle loss.

We evaluated the use of standard nose-only collectors to sample the mice simultaneously and nose-only ventilation masks for a single swine. The device was optimized initially for delivery of respirable particles, not collection. As demonstrated in Fig. 5b, before modification of the system, 32% of the carbon particles penetrated from an animal port to the entrance of the cold trap. Penetration was 82% after modifications that included inverting the tower, reversing airflows, and straightening sampling lines. Similar optimization and efficiency measurements are under way using nose-only ventilation masks for a single swine, discussed below.

Evaluation

Using the prototype collector design for piglets, we began collections from 2- to 4-week-old piglets. The prototype consisted of a ventilation mask adapted with a low-flow one-way valve to restrict contaminants present in the room air. The condenser system consisted of two 50-mL centrifuge tubes immersed in dry ice and linked in series. Directional airflow was maintained by intermittent application of vacuum during the collection. Piglets were injected intravenously with phosphate-buffered saline as a control or 150 μg/kg staphylococcal enterotoxin B (SEB). Approximately 2 to 3 mL of EBC was collected for 30 min at various times following exposure to SEB. EBC samples were evaluated for protein concentration, by ELISA assay, and by mass spectrometry.

As early as 1 h following treatment, we were able to detect a large peak at 17495.3 Da (m/z) in unprocessed samples (Fig. 6). This peak corresponds to the observed

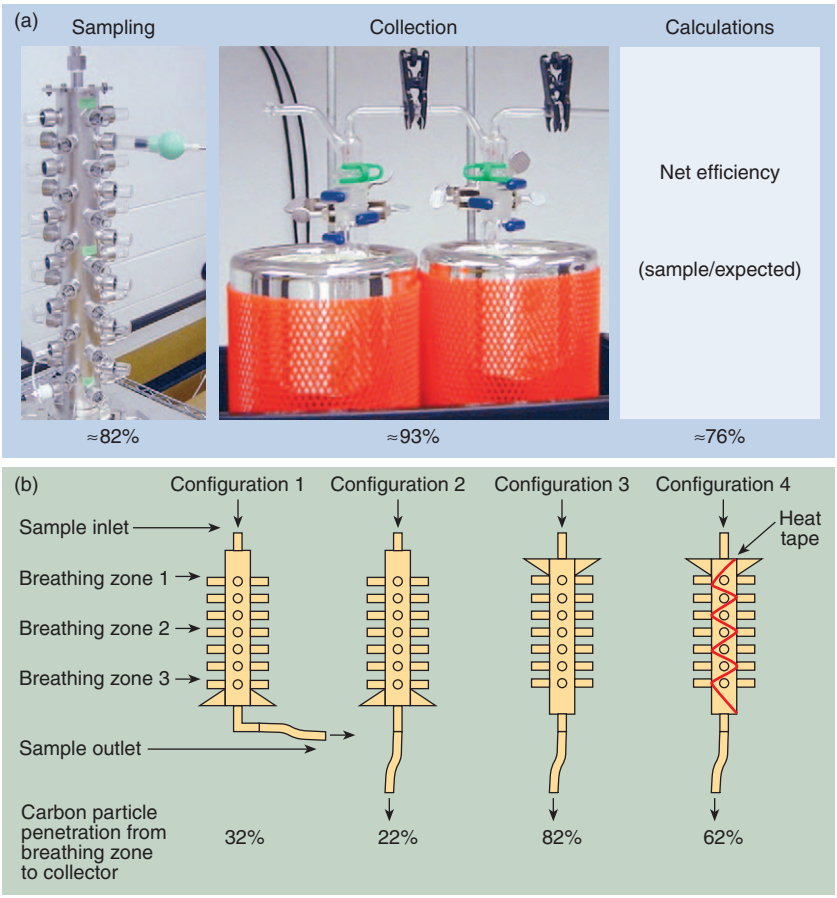

Figure 5. Design and collection efficiency of EBC collector. (a) The collection tower and condenser setup are pictured. The tower is designed to accommodate up to 50 animals in a single experiment. Numbers appearing below the figures represent the average efficiencies determined for each portion of the collector using 13-nm-dia. carbon black particles. (b) A schematic of four different collector configurations is shown. Airflow direction is indicated by the short arrows. Configuration 1: airflow is oriented in the same direction as used for aerosol exposures, and the outflow tube is oriented at a 90° angle. Configuration 2: airflow is oriented in the same direction as used for aerosol exposures, and the outflow tube is parallel with the airflow. Configuration 3: airflow is inverted relative to direction as used for aerosol exposures. Configuration 4: airflow is inverted relative to direction as used for aerosol exposures, and the tube is heated externally to prevent excess condensation. Numbers below the schematic indicate efficiencies measured for each design using carbon black particulates.

size for purified IL-2 for swine. We detected IL-2 in the ample by ELISA as well. Other cytokines, such as TNFα and IL-1, were not detected by either ELISA or mass spectrometry. Further confirmation of the 17495.3-Da peak as IL-2 using liquid chromatography/mass spectroscopy is still under investigation.

Currently, we are also investigating the chronological patterning of cytokine production in response to infectious and noninfectious agents using *in vitro* systems. Although the data are very preliminary, it appears that infectious agents can be distinguished from one another based on the number and timing of cytokine production *in vitro*. Using a standard panel of four cytokines at three time points, we have found the pattern of induction to be relatively consistent and distinguishable for various bacterial agents. Further investigations are under way to determine the level of distinction that may be possible by comparing the chronological patterning of cytokine induction, class level (bacterial, viral, or toxin), level of Gram stain (positive versus negative), or bacterial group to which an agent belongs (e.g., proteobacteria, *Yersinia* spp.).

CONCLUSIONS

We have demonstrated the ability of mass spectrometry to detect and resolve multiple cytokines in a single sample based on absolute mass. Resolution of multiple cytokines is not possible with other systems such as ELISA or PAGE in a single assay.

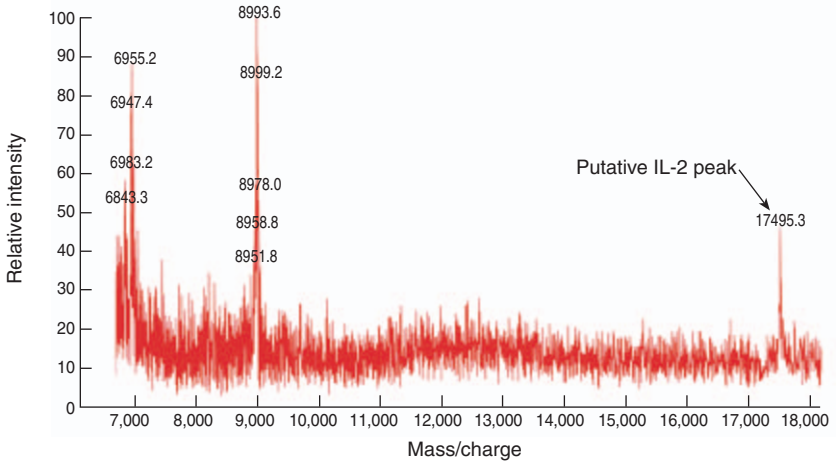

Figure 6. Mass spectral patterns of EBC from SEB-exposed piglets. Breath was collected and interrogated by MALDI TOF for the presence of unique peaks. The *x* axis is mass in daltons over charge (+1, +2, etc.) and the *y* axis represents the relative intensity of the peak. As shown, several significant peaks were observed in the 6,800–19,000 *m*/*z* range, where the peak at 17,495.3 *m*/*z* correlated with the peak expected for the singly charged IL-2. This peak was not seen in control samples. This result correlates with detection of IL-2 cytokine by ELISA assays conducted on the same samples.

Using these conditions, we have assembled a library of mass spectral signatures of host immune factors from multiple species.

We have demonstrated the ability to collect and detect cytokines in EBC after exposure to biological warfare or bioterrorism agents. Collector designs were evaluated for the ability to collect particulates and are being evaluated with aqueous samples containing known proteins. The prototype collection system has a mask that is adapted to restrict entry of room air and provides directional airflow through redundant condensers. Aqueous samples were introduced directly into the MALDI TOF mass spectrometer for analysis. Conditions were optimized using purified cytokines and various sample matrices. A single protein peak at a mass associated with swine IL-2 was rapidly detected in EBC from pigs. This peak correlated with masses observed for species-specific cytokines in our mass spectral library. The peak was specific to exposed piglets, and the presence of IL-2 was confirmed by ELISA assays.

We intend to further our design and analysis of EBC for the diagnosis of biological infection as well as a dosimeter for exposure to chemical and radiological insults.

REFERENCES

[1]Oppenheim, J., and Feldmann, M., *Cytokine Reference,* Academic Press, New York (2001).
[2]Standiford, T. J., Strieter, R. M., Greenberger, M. J., and Kunkel, S. L., "Expression and Regulation of Chemokines in Acute Bacterial Pneumonia," *Biol. Signals* **5**, 203–208 (1996).
[3]Standiford, T. J., Kunkel, S. L., Greenberger, M. J., Laichalk, L. L., and Strieter, R. M., "Expression and Regulation of Chemokines in Bacterial Pneumonia," *J. Leukoc. Biol.* **59**, 24–28 (1996).
[4]Callard, R. E., and Gearing, A. J. H., *The Cytokine Fact Book*, Academic Press, New York (1994).
[5]Vaddi, K., Kelle, M., and Newtown, R. C., *The Chemokine Factbook*, Academic Press, New York (1997).
[6]Reilly, M. P., Lawson, J. A., and FitzGerald, G. A., "Eicosanoids and Isoeicosanoids: Indices of Cellular Function and Oxidant Stress," *J. Nutr.* **128**(Suppl. 2), 434S–438S (Feb 1998).
[7]Cotter, R. J., "Time-of-Flight Mass Spectrometry: An Increasing Role in the Life Sciences," *Biomed. Environ. Mass Spectrom.* **18**, 513–532 (1989).
[8]Kunke, S. L., "Th-1 and Th-2 Type Cytokines Regulate Chemokine Expression," *Biol. Signals* **5**, 197–202 (1996).
[9]*Cytokine Reference Vol. 1: Ligands*, J. J. Oppenheim and M. Feldman (eds. in chief) and S. K. Durum, T. Hirano, J. Vilcek, and N. A. Nicola (eds.), Academic Press, San Diego, CA (2001).
[10]Balkwill, F. R., *Cytokines—A Practical Approach*, Oxford University Press, New York (1991).
[11]Ramshaw, I. A., Ramsay, A. J., Karupiah, G., Rolph, M. S., Mahalingam, S., and Ruby, J. C., "Cytokines and Immunity to Viral Infections," *Immunol. Rev.* **159**, 119–135 (1997).
[12]Chamberlain, A. C., and Dyson, E. D., "The Dose to the Trachea and Bronchi from the Decay Products of Radon and Thoron," *Brit. J. Radiol.* **29**(342), 317–325 (1956).
[13]Fairchild, C. I., and Stampfer, J. F., "Summary Report: Particle Concentration in Exhaled Breath," *Am. Ind. Hyg. Assoc. J.* **48**(11), 948–949 (1987).
[14]Lu, D., Stanley, C., Nunez, G., and Frazer, D., "A Mathematical Description of Pressures in Alveolar Pores of Kohn," *J. Biomech. Eng.* **113**(1), 104–107 (1991).
[15]Suki, B., "Fluctuations and Power Laws in Pulmonary Physiology," *Am. J. Respir. Crit. Care Med.* **166**, 133–137 (2002).

ACKNOWLEDGMENTS: This work was supported by the Defense Advanced Research Projects Agency (MDA972-01-D-0005). We would like to thank Earl Tewksbury for generating and sampling nano-aerosols and Nathan Boggs for collection of samples and critical review.

THE AUTHORS

JOANY JACKMAN has a Ph.D. in cellular and molecular biology from the University of Vermont. She came to APL in June 2000. Formerly involved in research examining cell cycle regulation in cancer, she studied the role of infectious disease in those processes at the National Cancer Institute. Dr. Jackman became involved in biological warfare defense as an Independent Public Agent at the U.S. Army Medical Research Institute of Infectious Diseases. She has continued her work in developing, improving, and evaluating reagents, devices, and technologies for reducing biological threats to the military and civilians. Her e-mail address is joany.jackman@jhuapl.edu.

OWEN MOSS received a Ph.D. in inhalation toxicology and a master's degree in radiation biology from the University of Rochester School of Medicine and Dentistry. His bachelor's degree in physics is from New Mexico State University. Dr. Moss worked at Los Alamos National Laboratory and at Battelle Pacific Northwest Laboratory prior to his current position at CIIT Centers for Health Research in Durham, North Carolina. His research interests over the past two decades have focused on mechanisms that relate particles and biomarkers of inhaled particles to human health. Dr. Moss has contributed to more than 60 peer-reviewed publications and holds 9 patents. His e-mail address is moss@ciit.org.