Host Gene Expression Responses to Biothreat and Infectious Agents: Implications for Mathematical Modeling of *in vitro* Responses

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NATO CONFERENCE: OPERATIONAL ISSUES IN CHEMICAL AND BIOLOGICAL DEFENSE

Introduction: Detection of exposure to biological threat agents currently uses culture methods, immunoassay and gene amplification and these methods constantly are being perfected for greater sensitivity. However, recent events have demonstrated that assessing exposure to a biological threat agent well in advance of onset of illness or at various stages post-exposure would be an important capability to have among the diagnostic options. There is an urgent need for better diagnostic tools that will be sensitive, rapid and unambiguous to identify pathogen exposures and aid in triage of affected personnel. Rationale: Patterns of host cell transcription offers the potential for diagnosis of exposure/infection at a very early stage, even as early as ~1 hour post-exposure. The ability of diagnostic assays to identify infected individuals during the prodromal period is essential for successful treatment or intervention following exposure and infection with many disease-causing agents. The identification of early markers of infection offers great promise for revolutionizing disease diagnosis.

We have used gene microarray technology to characterized host gene expression responses (rather than direct pathogen exposure) to 14 biothreat and infectious agents using ex vivo exposures to each pathogen in peripheral blood mononuclear cells (PBMC). We found sets of genes that readily identify the pathogen used. By a thorough evaluation of healthy human baseline gene expression (using 75 diverse individuals), we identified genes with unambiguous responses for pathogen exposures. For 3 of these pathogenic agents, we have also carried out in vivo exposures in non-human primate models. Gene responses determined from blood drawn at various time periods post-exposure show clearly recognizable patterns of gene expression at remarkably early time frames. For example, B. anthracis exposure can be detected by direct pathogen identification at ~72 h post-exposure, while profiling of host gene expression responses identified an anthrax-specific pattern by at least 24 h. Due to the difficulties of such animal studies, we utilized bioinformatics approaches to correlate the in vitro to the in vivo gene responses and identified gene sets from in vitro studies that correctly predict pathogen exposure in vivo. Using the in vitro system at multiple concentrations and time periods post-exposure and mathematically modeling the gene responses to predict in vivo exposure patterns for those variables offers a means to establish a comprehensive approach for use of host gene expression responses for diagnostic purposes.

*Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition.

**Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

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1. INTRODUCTION

The elucidation of the genetic events underlying the initiation and progression of human diseases is a critical step in designing better tools for diagnosis and treatment, and the advent of high-density microarray technology provides incredible opportunities for studying the disease process. High-throughput DNA and tissue microarray techniques promise to revolutionize the discovery and validation of novel molecular markers. Based on the information on the genome sequence as the outcome of the human genome project, functions of genes and proteins have been studied by expression profiling using microarrays, proteomics, single nucleotide polymorphism analysis, and coupled with bioinformatics. Along with advances in pharmacogenomics, these studies have raised the prospect of developing tests for individualized medicine based on genetic information, such as those predicting a person's susceptibility to diseases, or responsiveness to drugs for choice of treatment.

1.1 Global Molecular Analysis

Use of global methods that allow for high-throughput assessment of cellular function have become widely used by clinical and basic scientists working in both academia and the pharmaceutical industry. Differential display (DD)-PCR, DNA microarray, serial analysis of gene expression (SAGE), and subtractive hybridization are some of the techniques used to study global gene changes in numerous cell systems. The most efficient of these, microarray techniques, permits screening of ~40,000 genes simultaneously, producing information in an efficient manner. These are robust techniques, designed to highlight genes of interest that can be identified based on changes in expression profiles relative to a series of appropriate controls. Confidence levels are improved in studies that utilize sufficient numbers of replicates (4 experimental replicates, and having genes in duplicate on each slide, have been recommended for use in the microarray statistical package, Partek Pro, St. Louis, MO) and focus on clearly identified changes. For example, genes that have altered expression levels >5-fold, are reproducible in replicate samples. Similarly, genes having >3-fold changes are similarly altered in quite high percentages of the replicates. Focusing on the genes that have major changes in expression is a useful approach for selecting genes of interest for diagnostic purposes, but for therapeutic and mechanistic studies, important genes could be overlooked, since some minor change in gene expression level may result in a broad upstream change. Upon selection of genes of interest, complementary confirmatory techniques (RT-PCR, real-time-PCR, etc) are used to verify the response profiles. All of these techniques for global gene analysis are quite laborious, however, the resultant information obtained using these techniques is of incredible value for designing innovative diagnostic and therapeutic approaches. Studies utilizing each of these techniques have identified gene profiles that have been the basis for major advances in medical approaches

1.1.1 Current Uses Of Microarrays For Identification Of Unique Gene Patterns

Current publications show examples of the use of microarray technology for identification of differentially expressed genes in several sets of cancers (7-10). Cohen et al (11) have used this technology to identify genes induced by Listeria monocytogenesis in human promyelocytic THP1 cell line. Brutsche and colleagues (12) used peripheral blood mononuclear cells to study human B-cell isotype control and IgE production in patients with atopy and asthma and found significantly altered expression relative to healthy subjects. For several of these clinical studies, the number of patients enrolled are rather large (~100 for cancer studies) and ~20 for the asthma study; this number provides adequate statistical strength for correlation of gene patterns for differentiating the specific disease state

2. APPLICATION TO PROBLEMS OF IMPORTANCE FOR BIOLOGICAL DEFENSE

The threat of clandestine terrorist action using biological or infectious agents is especially a problem since many of these pathogens elicit non-specific flu-like symptoms early in infection; therefore, exposed individuals may not seek medical attention as a treatable stage. In contrast, an overt hoax or actual attack could result in people undergoing undue panic and medication during the long process of attempting to identify the pathogen.

Detection of exposure to biological threat agents currently uses culture methods, immunoassays and gene amplification, and these methods are constantly being perfected for greater sensitivity. However, recent events have demonstrated that assessing exposure to a biological threat agent well in advance of onset of illness, or even at various stages post-exposure would be an important capability to have among the diagnostic options. The ability of diagnostic assays to identify infected individuals during the prodromal period is essential for successful treatment or intervention following exposure or infection with many diseases-causing agents. The identification of early markers of infection offers great promise for revolutionizing disease diagnosis.

2.1. Goals of the Research Efforts in Applying Microarray Technology to Biodefense Problems

The ultimate goal of this research effort is to derive a series of gene expression responses to biological threat agents for use as diagnostic markers for exposure, as well as to analyze the information to design stage-specific therapeutic regimens. The rationale behind this approach encompasses several different scenarios.

2.1.1 Direct Pathogen Identification

It is frequently very difficult to identify an actual pathogen when a person presents with non-specific symptoms or if he/she "thinks" there might have been an exposure. Actual pathogen detection depends on its concentration in the matrix being examined, and that detection threshold may be reached too late for effective intervention in the case of many biological threat agents. Furthermore, when visiting a physician for even common illnesses, throats are swabbed and cultures are taken, and often a pathogenic agent is not identified, but rather a "footprint" that suggests a possible source of the infection is deduced. This presents difficulties when dealing with biological threat agents since many of these illnesses will not subside so readily as would be expected with common illnesses, such as influenza. Furthermore, many biothreat-induced illnesses start with flu-like symptoms, have varying latent periods and most proceed to cause lethality if countermeasures are not initiated early post-exposure (Figure 1).

Biological threat agents show different kinetic patterns of illnesses even though many start out with flu-like symptoms. In general, toxins are fast acting, while bacteria and viral exposures can vary in illness onset from days-months. Therefore, timing of exposure is very important; for each of these studies we characterized gene changes at early, mid, and late time points to understand the kinetics and pathogenesis of the illness. In our studies, we have observed some sets of genes that remain changed throughout the experiment, while other genes may show altered regulation at specific time frames post exposure.

2.1.2 Rapid Responses Are Essential For Biothreat Detection.

Early detection is very important to successfully treat exposure to biological threat and infectious agents. As was pointed out, actual pathogen detection is concentration-dependent and would most likely not reach sufficient levels in the early stages (13, 14). In our current work, in which we have exposed NHP to B. anthracis, the animals did not begin to how signs of even mild flu-like illness until 3-4 days. By 24 h, the

earliest time period examined in vivo, a robust gene expression response showed unique gene profiles (15). We are presently examining earlier time periods and believe that identification could confirm B. anthracis exposure even as early as 6-12 h, since the 24 h time period showed a robust and specific response

2.1.3 Unidentifiable Pathogens/ Bioengineered Agents.

One concern expressed by military experts relates to the fact that due to the biological sophistication of terrorist groups, chimera, mutants (natural or deliberate), or other modified pathogenic agents could escape detection if assayed with structural-based probes. We observed that certain sets of gene expression responses appear to be related to a "course of impending illness". For example, we have seen that "shock-inducing pathogenic agents" have common responses, and data mining shows a relationship of gene patterns that could indicate altered regulation of vascular tone, vascular leakage, pulmonary distress, etc. Our data suggest that gene patterns can reveal a profile related to the course of impending illness. If such a pattern is identified, medical personnel could initiate treatments to avert disastrous results.

2.2 Ongoing Studies Applying Microarray Technology To Biological Defense Problems.

We have selected agents from three different classes of pathogenic agents, bacterial pathogens, viral pathogens, and bacterial toxins and studied their host immune response in human PBMCs. 2.2.1 Bacterial toxins: The studied toxins can cause onset of acute illness within a few hours, but side effects can persist for many weeks. Staphylococcal enterotoxin (SE) B, a superantigen, Botulinum toxin A, a neurotoxin and Cholera toxin, an enterotoxin, each cause severe illness rapidly (Figure 1) and can proceed to death within a few days (13).

2.2.1 Biological Threat Agents Studied:

Bacterial. *Bacillus anthracis*: Inhalational Anthrax occurs once spores germinate in alveolar macrophages and are transported to the mediastinal lymph nodes. *Brucella melitensis*: Brucellosis is caused by gram-negative bacteria (B. melitensis), and is highly infectious via an aerosol route. *Yersinia pestis* causes pulmonary plague, which is fatal within a few days if left untreated (13). *Viruses:* Two RNA viruses have been studied for their induction of gene changes in human lymphoid cells. Venezuelan equine encephalitis (VEE) and Dengue infections each initially induce intense flu-like illness including headaches, fever and malaise. CFR could be a range from <1% (for dengue as well as for VEE) to in excess of 30% for dengue hemorrhagic fever. *Common illnesses*: Studies of febrile illnesses, influenza, adenoviruses and other illnesses are ongoing in various laboratories, including our own. Since so many biological threat agents begin with flu-like illnesses, it is extremely important to characterize these common illnesses so that genes can be selected that will unambiguously differentiate biothreat agents from these other febrile illnesses. Ongoing studies in other labs include gene profiling with samples from rheumatoid arthritis patients, general febrile illnesses and other illnesses that cause inflammatory responses.(16).

2.2.2 Bioinformatics and Data Mining:

As we started working on gene expression patterns we soon realized how important it was to have strong database and bioinformatics support. Additionally, we adapted or developed software necessary for analyzing, sorting, clustering and subjecting our data to statistical parameters. We have now accumulated increasingly more complex software from commercial sources for these analyses.

Our studies were carried out with 2-4 replicates of each of the 3-5 exposure times per pathogenic agent to provide extensive statistical strength. Furthermore, we have applied high-powered algorithms, designed to evaluate such massive data sets and focus on gene patterns that can identify gene patterns that will differentiate among the various pathogenic groups. In addition, our laboratory has developed automated

data mining tools in order to retrieve published information that could provide functional relationship links for the corresponding proteins (of genes significantly altered). We are collaborating with mathematicians who are independently scrutinizing the data and designing predictive modeling approaches.

2.3 Specific Findings In Our Laboratory:

Our objective was to create a library of host gene expression responses upon exposure to biological threat agents (15, 17). We first carried out these studies in vitro, exposing PBMC from healthy donors to the selected pathogen for various time periods, based on the experience of the pathogen specialist monitoring the course of the infection. We then selected 2 pathogenic agents for which NHP exposure closely replicates the illness as observed in humans. After aerosol exposure in NHP to these selected pathogenic agents, PBMC were collected at various time periods post-exposure and the resulting gene profiles determined. We find it necessary to use both in vitro and in vivo approaches for several reasons. First, there are some pathogenic agents for which an appropriate animal model does not exist. Secondly, extensive investigations of dose/time need to be carried out in vitro and selected information confirmed in vivo due to the costs and concerns associated with use of NHP. We expect that it may be very important to utilize species closely related to humans since certain differences in organ function are seen between rodents and humans. Indeed, we have rodent-specific gene arrays that are used for other studies, but we believe rodent models would not necessarily be appropriate for this objective. It is likely, though, that in vitro studies for such pathogenic agents may be the most reliable data that can be obtained and therefore, we are collaborating with mathematicians to develop sophisticated mathematical simulations with predictive modeling to provide important projections for these applications.

2.3.1 in vitro Studies:

For 8 pathogenic agents, we have compared gene patterns, sorting genes based on their function, and found that most pathogens upregulated genes coding for inflammatory mediators. This is not surprising and actually confirms what we know about the action of these biological threat and infectious pathogenic agents. Although inflammatory mediators may not differentiate among threat agents, they may best serve as markers indicating the progression of illness. There are many options for sorting and clustering the data utilizing specialized bioinformatics approaches; applying these techniques identified unique gene patterns induced by each agent. For some pathogenic agents, ~20 genes created a signature that was unique from the other 7 pathogens. Many of these genes relate to cell surface receptors and other molecules that the pathogenic agent utilizes to initiate its invasive action as well as to specific signaling sequences utilized by the pathogen.

2.3.2 in vivo Studies:

We selected 2 threat agents (B. anthracis and SEB) for which the NHP model closely reflects the illness induced in humans so that we could observe the type of gene patterns from in vitro studies that would likely be replicated in vivo (13, 18). Although the gene responses seen in vivo are very robust, encompassing both primary and secondary events, there are selected patterns that are remarkably replicated in vivo vs in vitro; these tend to relate to lymphoid receptors and the subsequent signaling cascades induced by each of these unique pathogenic agents. An example is a specific G-protein upregulated by SEB in NHP. It was not seen above background levels in our group of 75 healthy control individuals. For in vitro studies, the kinetics of this gene pattern is remarkably unique for SEB exposure. In this figure, the over-expression response was obvious in 30 min (3 h before onset of vomiting) and it persisted through at least 24 h (the last time period examined). For SEB, death from lethal shock is usually seen from ~42-60 h in NHP and information from accidental exposures of humans indicates a comparable time frame. Similarly, for B. anthracis exposure in NHP, unique sets of genes that show specificity for anthrax were identified even by 24 h post exposure, 2 days prior to identification of the

pathogen in body fluids and ~4-6 days prior to the onset of clinical signs of illness. Interestingly, we found genes that were early indicators of early exposure (24 h) vs later progression (72 h). This finding suggests that the "dose/time" combination may be able to be estimated based on gene patterns. These gene sets would not necessarily be the ones chosen as the markers of anthrax or other pathogen, but rather as staging indicators.

Biological threat agents show different kinetic patterns of illnesses even though many start out with flu-like symptoms. In general, toxins are fast acting, while bacteria and viral exposures can vary in illness onset from days-months. Therefore, timing of exposure is very important; for each of these studies we characterized gene changes at early, mid, and late time points to understand the kinetics and pathogenesis of the illness. In our studies, we have observed some sets of genes that remain changed throughout the experiment, while other genes may show altered regulation at specific time frames post exposure.

2.3.3 Therapeutic Implications.

As mentioned, several of these pathogenic agents eventually result in lethal shock and so we focused our attention on genes known to regulate vascular tone or contribute to vascular leakage. We then carried out analyses to determine what other genes co-regulated with known modifiers of the vasculature, focusing on genes whose expression level was altered preceding and following appearance of vascular lesions in the animal models. This led to some interesting findings relating to gene sets that may predict a "course of impending illness" and could provide the basis for designing stage-appropriate therapeutic intervention strategies. We are obtaining clinical samples from various "common" illnesses to further confirm that the gene patterns selected identify a specific biological threat or infectious agent. Another study in our lab involves determination of the effects of severe physical stress (to approximate battlefield conditions) on host gene expression responses to some of the same pathogenic agents that we have already studied.

2.3.4 Gene Profiles As A Baseline For The Health Human:

Our wealth of data obtained from PBMC gene profiles for 75 healthy human volunteers (diverse in ethnicity, sex and age) identified genes that are expressed at background levels in the healthy subjects but become massively overexpressed in response to one or more pathogenic agents (15, 17). These genes may provide unambiguous markers that could readily differentiate exposure to a pathogenic agent from unexposed individuals since the differences between "baseline vs exposure" is reproducible and easily obvious, despite ethnic/sex/age diversity. The functions of these genes, in general, relate to severe cellular responses to agonists/antagonists. We have confirmed altered regulation of selected genes using RT-PCR.

2.4 Gene Profiles Showing Unique Patterns For Specific Biothreat Agents

Figure 2 shows an example of a few selected genes that were expressed at near-background levels in the 75 healthy human volunteers, but show a unique pattern upon exposure to Brucella. As this graph illustrates, any 2 agents may show similarities in gene responses for some genes, the overall pattern is not the same when a sufficient number of genes are used for the indicators. A number of mathematical tools are used to select the specific genes and the number of genes needed to differentiate one pathogenic agent from the others. For example, using the Bonferroni analysis (19), 9 genes were identified as the minimal number to differentiate SEB from CT. Similarly, application of this approach to 4 of these pathogenic agents identified 25 genes as the minimum number to distinguish among those pathogenic agents.
Infectious Agents

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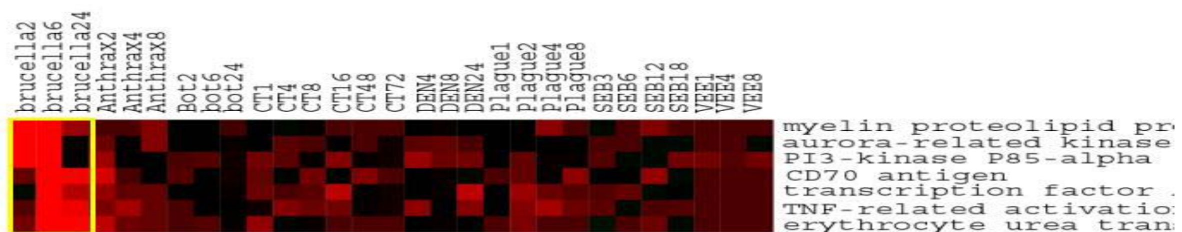

Figure 1. RT-PCR confirmation of selected gene responses initially identified from global gene analysis studies. In this graph, bright red indicates the highest upregulated genes. These genes were selected for this study since, for the 3 time periods of exposure to Brucella, they displayed massive up-regulation. Although there are some sporadic increases in these genes upon exposure to the other pathogenic agents, use of even these genes would enable one to discriminate brucella from the other pathogens. Our intention is to select ~50 such genes (which can be overlapping) to characterize each pathogenic agent

2.3.6 Studies From Other Laboratories

The Relman group has shown that there are distinct patterns of gene expression induced by Gram-negative and Gram-positive bacteria (16). They also compared heat killed isolates of Bordetella pertussis, E. coli, S. aureus and found gene patterns related to function to be altered in human PBMC. That laboratory has extensively focused on establishing profiles for "healthy" responses. DNA microarray has been used recently to report gene changes induced by various pathogens that give a unique gene response in dendritic cells (20). Other investigators are focusing on establishing gene profiles to characterize exposures to chemical warfare agents.

2.4 What are the Unique Problems Associated with Using Gene Arrays for this Purpose

What are the unique problems associated with using gene arrays for this purpose.Exposures to biothreat agents seldom occur in humans and therefore, such studies must rely on animal models that closely mimic the illness as it has been described to occur in humans. This usually involves use of non-human primates, and this is in contrast to many of the gene discovery studies previously described, in which the diseases

commonly occur throughout the general population. Furthermore, for some threat agents, representative animal models of the human illness are not available and so in vitro studies become the only alternative. Therefore, for host responses to biothreat agents, we first determined gene profiles upon exposure to peripheral blood mononuclear cells from healthy donors in vitro, and then compared those results with profiles obtained from in vivo exposure of non-human primates to the same biothreat agents

2.4.1 Kinetic Considerations

Gene patterns show time-related responses and Figure 1 illustrates the differences/similarities in onset of illness from the threat agents. During a terrorist attack, personal exposure dose would be unknown and time post-exposure could be unknown. One question we are attempting to answer relates to the findings in Figure 2, in which we demonstrate that if dose is constant, one can track time (post exposure) by selecting the secondary response gene profiles. We want to know if increased dose shows a similar change in gene profiles and if that change is reflected with essentially common gene sets to possibly indicate the "progression of illness."

2.4.2 Microarray Related Considerations

With the considerable amount of data generated by the different technologies using microarrays, it is obvious that the reading of the information and its interpretation and management through the use of bioinformatics is essential. Various techniques for data analysis are currently available and they require extensive evaluation before adapting to each project. Biochip and microarray technology have an essential role to play in the evolving trends in healthcare, which integrate diagnosis with prevention/treatment and emphasize personalized medicines.

2.4.3 Bioinformatics

Microarray data, by sheer virtue of their volume, can be better interpreted and analyzed using good statistical methods. Having replicates to validate and statistical analysis to define what is significant change is another critical issue in the field of microarray. Statistical analysis requires numerous replicates, which could sometimes be an issue with patient samples, monkey samples or experiments using rare pathogens. There are volumes that have been written regarding data processing, and we suggest those sources (21-29). Of course, some of the "noise" among samples involving patients relates to genetic variation, single nucleotide polymorphism (SNP) etc (30). Our studies have sought to diminish the noise from these factors by analysis of the unstimulated cells from 75 healthy volunteers (diverse in sex, age, ethnicity).

Another problem that needs some attention is how to translate these findings into a meaningful simple assay that can be performed by minimally trained personnel. In order to find a solution we decided to identify genes that are never expressed in normal healthy individuals but were massively overexpressed upon exposure to one or more pathogenic agent. The dramatic change in the expression levels aid in avoiding ambiguity.

We also believe that for the present time, the massive gene arrays are a preferred research laboratory tool, rather than a rapid clinical diagnostic. There are a many companies that aim to assess ~1000 genes simultaneously in nearly real-time and that approach offers good possibilities for early use of this technology in military efforts.

2.6. Conclusions and Future Plans.

In the past several years, our studies of host immune responses to biological threat agents have centered on signal pathways, cell mediators, and evaluation of gene responses upon exposure to biological threat

agents both in vitro and in vivo. Peripheral blood lymphoid cells can serve as a reservoir of historical information and are readily obtained from an exposed individual. We have shown that each pathogenic agent induces gene changes that are unique to that agent. These gene changes can be used for diagnostic markers as well as targeted for therapy.

By selecting genes that are expressed at low-levels in healthy volunteers yet show massively altered regulation upon exposure to one or more biothreat agents, we can establish gene patterns indicative of a particular agent, degree of exposure, etc and interpretation difficulties will be minimized. Similarly, we have also found a series of genes that are expressed at very high levels in these unstimulated samples, some of which become severely down-regulated upon exposure to one or more of the pathogens.

The studies thus far show specific gene profiles for the various biothreat agents that occur soon after exposure and these molecular changes in the PBMC appear far ahead of the actual symptoms or signs of illness, therefore, detection of a specific pattern early enough will permit these genes to be used as diagnostic and possibly therapeutic markers. We do intend to extend our studies to identifying distinct protein profiles induced by various biological and infectious agents, since they have shown interesting possibilities in detection of cancers (31). Use of such gene and protein screening techniques will give a new parameter to design new therapeutic approaches and design diagnostic tests for detection at any stage of the disease.

Microarray technology has important applications in pharmacogenomics: drug discovery and development, drug safety and molecular diagnostics. DNA chips will facilitate the integration of diagnosis and therapeutics, as well as the introduction of personalized medicines.

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SYMPOSIA DISCUSSION - PAPER 20

Authors Name: Dr Jett (US)

Discussor's Name: LtCol Saracci (TU)

Question:

1a) Do you believe that host response-based methods would replace non-cultured, agent-based amplification methods, such as PCR?

1b) Especially, respect to the high sensitivity of these amplification methods.

2) How can you make discriminations among micro-organisms which use same/similar pathogenic mechanisms in host, probably causing similar host responses?

Author's reply:

1a) All methods will have an important miche. I do not expect host-responses to replace, but rather supplement other methods.

1b) Even at high sensitivity, the best PCR amplifications must have the pathogen to result in a signal. It takes time before even any pathogen can be detected in (small amounts of body fluids)

2) We can discriminate among very closely related pathogens (such as staphenrerotoxim A & B). This discrimination may relate to receptors G – proteins and signals although the eventual outcome is vascular leakage, leading to DIC, multi-organ failure and death. We have seen vast differences in gene responses to LPS, SEA, SEB especially relating to CD markers, G Proteins, etc. Inflammatory medicators do not easily discriminate and we would not expect them to do so. However, they show the severity of the exposure and "stage" of impending illness. Although therapy targeting antictokine effects has been shown in the literature (Karima – 1996) to not have a significant effect on outcome. Other therapeutic targets (microemboli using anti thrombin or the Eli Lily drug, Xigris) may be common, but the timing is different for each shock – inducing pathogen.

Authors Name: Dr Jett (US)

Discussor's Name: Capt (USN) Campbell (US)

Question:

Dr. Ken Olden, NIEHS (US) started an initiative called "The Environmental Genome Project" to identify Human Genes that conferred resistance of susceptibility to environmental threat. Are you working with NIEHS to measure expression of any "Environmental Genes" that their project identifies?

Author's Reply:

I have lost contact with NIEHS, and that is regrettable.

I am so pleased that you brought up this point and I will pick up that thread again. The funding from the NIEHS Project will be important, especially when evaluating "Project Normal" among humans.