IDENTIFICATION OF CHANGES IN GENE EXPRESSION INDUCED BY TOXIC AGENTS: IMPLICATIONS FOR THERAPY AND RAPID DIAGNOSIS.

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NATO Conference: Operational Issues in Chemical and Biological Defense
Human Factors in Medicine Panel
SESSION IV-BIOLOGICAL DEFENSE
Presentation #19

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	2001	Final , Proceedings, 2001

4. TITLE AND SUBTITLE
Identification of changes in Gene expression induced by toxic agents: Implications for therapy and rapid diagnosis
Proceedings of NATO conference: Human Factors in Medicine Panel, May 2001.

5. FUNDING NUMBERS
H961

6. AUTHOR(S)
Marti Jett, Rina Das, Christiano Cummings, Chanaka Mendis, Roger Neill, David Hoover, Luther Lindler, Chrysanthe Paranavitana, Xiaozhe Huang, George Ludwig, Erik Henchal, David C.H Yang (2001).

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Division of Pathology
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
DARPA
Arlington, VA

U.S. Army Medical Research and Material Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

In our changing world, there has been a significant increase in both the nature and degree of the threat posed by the use of biological agents. Studies for many years have focused on rapid detection of known biological threat agents using structural-based probes designed and directed toward features of the pathogenic agent. However, concerns relating to unidentifiable pathogens, that could result from either deliberate or natural mutation processes have prompted studies to find alternative approaches. Our thesis was that an exposed individual would show gene expression responses unique to the pathogenic agent and prior to onset of the full illness. Therefore this study focused on use of peripheral blood mononuclear cells (PBMC) as a readily accessible reservoir of historical information for development of a library of host gene expression responses to known biological threat agents. The gene responses seen in this accessible tissue would be a compilation of both primary and secondary effects on PBMC and would present a signature pattern of a specific biological threat agent. This study describes our work to establish a library of host responses to pathogenic agents for use to a) predict the course of impending illness especially for unidentifiable pathogens so that appropriate therapeutic intervention can be initiated, b) to characterize the degree of individual exposure in order to assist health personnel to rapidly differentiate those who will become seriously ill from "the worried well" individuals and c) reveal new therapeutic targets that can be initiated even in late-stage illness caused by biological threat agents.

14. SUBJECT TERMS
Biological warfare agents, Host responses, gene expression pattern, Anthrax, VEE, Brucella, Plague, Staphylococcal enterotoxins, Diagnostic marker genes, DNA chip, lymphoid cells.

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
unclassified	unclassified	unclassified	unlimited

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ABSTRACT

In our changing world, there has been a significant increase in both the nature and degree of the threat posed by the use of biological agents. Studies for many years have focused on rapid detection of known biological threat agents using structural-based probes designed and directed toward features of the pathogenic agent. However, concerns relating to unidentifiable pathogens, that could result from either deliberate or natural mutation processes have prompted studies to find alternative approaches. Our thesis was that an exposed individual would show gene expression responses unique to the pathogenic agent and prior to onset of the full illness. Therefore this study focused on use of peripheral blood mononuclear cells (PBMC) as a readily accessible reservoir of historical information for development of a library of host gene expression responses to known biological threat agents. The gene responses seen in this accessible tissue would be a compilation of both primary and secondary effects on PBMC and would present a signature pattern of a specific biological threat agent. This study describes our work to establish a library of host responses to pathogenic agents for use to a) predict the course of impending illness especially for unidentifiable pathogens so that appropriate therapeutic intervention can be initiated, b) to characterize the degree of individual exposure in order to assist health personnel to rapidly differentiate those who will become seriously ill from "the worried well" individuals and c) reveal new therapeutic targets that can be initiated even in late-stage illness caused by biological threat agents.

INTRODUCTION

Identification of pathogenic agents using structural-based probes directed at specific pathogen properties has been the classic approach for rapid detection of biological threat agents. There are situations in which that system could need a supplemental approach. The obvious

scenario would be pathogenic agents that are unidentifiable due to deliberate or natural mutations. In addition to that unique situation, even for identifiable bacterial pathogens, extent of individual exposure would be limited by detection thresholds. This relates to the findings that a) some toxins are sequestered into target tissues and unavailable for identification in blood samples or b) bacterial pathogens require time to undergo sufficient growth to reach threshold levels for detection. For example, detection of bacterial products in human blood samples after exposure to B. anthracis have been found 2-3 days post exposure and by that time, the pathogen had undergone extensive proliferation, and serious illness was manifested. Similarly, for staphylococcal enterotoxin (SE) B, the toxin disappears from the peripheral circulation and is sequestered in the kidney (~70%) and other organs within 30 min post exposure. Identification of the actual toxin/fragments in blood or urine has not been seen unless blood samples were taken just following exposure.

The approach we have pursued relies on gene expression responses to biological threat agents using PBMC of the exposed individual and we are accumulating a library of these responses to infectious and biological threat agents. Although PBMC may not be the primary target for particular a pathogenic agent, they can respond to a combination of primary and secondary effects and they reflect information, in the form of secreted products as well as gene responses, related to stimuli they have encountered. Gene discovery technology provided the opportunity to examine large numbers of genes simultaneously for the various biological threat agents, both in vitro and in PBMC from animal exposed to the threat agents. Certainly, structural-based probes to identify biological threat agents offer the ability to test environment/personnel externally if an exposure is obvious and if the pathogen is identifiable. In contrast, the gene library we are developing is designed to relate gene profiles with subsequent illness patterns so that natural or deliberately modified pathogenic agents could be characterized and appropriate countermeasures initiated to ameliorate or prevent serious illness. In addition, examples of mass accidental exposures throughout the world can result in chaos at medical care facilities, due in part to panic(1). The gene array technology offers the potential to determine the degree of individual exposure (and perhaps susceptibility) so that the "worried well" could be separated from the seriously ill. This technology is undergoing phenomenal advances relating to devices that automatically process blood samples for isolation of RNA in minutes, and newly described technology to reduce PCR-based analysis of gene arrays to 30 min. Based on the developing library of gene responses to biological threat and other pathogenic agents, our ultimate objective is to design gene chips containing relatively few genes (hundreds rather than thousands) that could concisely predict the likely pathogenic agent or modified version of such, the degree of individual exposure and the course of impending illness. This approach aims to provide a tool for defense against biological threats so that resultant panic, morbidity, mortality can be reduced and targets identified for even late-stage treatment modalities.

METHODOLOGY EMPLOYED IN THIS STUDY

Overview. For the past 14 years, our laboratory has been carrying out in vitro and in vivo studies of host responses to staphylococcal enterotoxins as biological threat agents and those studies centered on signal pathways (2-6), cell mediators (5-8), and evaluation of gene expression responses(5). For the latter studies, we have specifically used differential display (DD) -PCR (5) and gene array analysis technology (9)to determine cellular responses to *B.*

anthracis, B. melitensis, Y. pestis, staphylococcal enterotoxins (SEs), cholera toxin (CT) and a number of other threat and infectious agents.

Gene discovery technology. There are numerous reports describing the use of global gene analysis to identify critical changes in expression of a few selected genes indicative of specific illnesses. For example, identification of the gene coding for Fetuin was reduced by 45% in liver cirrhosis (10) In some cases specific genes disappeared, such as i) Annexin VI expression in melanoma progression (11) or ii) MIF (macrophage migration inhibitory factor) in metastatic prostate cancer patients (12). These are just a few examples of the utility of gene array analysis to identify surrogate markers for a disease state and provide avenues for therapeutic intervention.

Gene microarrays. In the studies we describe in this article, we have utilized gene arrays/microarrays to define gene expression patterns for diagnosis as well as to identify potential new approaches for targeting therapy. Our first approach was to use large commercial screening arrays so that we could design inexpensive custom microarrays to define pathogens in terms of kinetics and dose responses. For in vitro studies, blood was drawn from healthy volunteers and PBMC were obtained by elutriation (13). PBMC were exposed to each biological threat agent (or control) for the designated time period, RNA extracted and purified, RT carried out and the resulting product hybridized onto the gene arrays according to standard procedures (9). The differences in gene expression between control and test for each gene was determined by specialized computer programs. The data were subjected to various software packages for statistical and cluster analysis (for genes within a time period and threat agent) aimed for these huge data sets (14, 15). Self-organizing maps and other analytical tools were applied to determine patterns of gene expression similarities and differences for each biological threat agent and these analyses also correlated information according to exposure time periods. For in vivo studies, blood was drawn, as described previously (16, 17), from exposed or control monkeys (other of our studies have used piglet models of SE-induced lethal shock) at the designated time periods. In these studies, PBMC were isolated and purified and the same procedure followed as for the in vitro experiments. This approach has provided information in an efficient manner and will facilitate development of a library of genes involved in pathogenesis for each agent examined.

Pathogens. PBMC exposure to each of the pathogens was carried out in the laboratory of the person who is the expert for each biological threat agent. The following is a list of the expert associated with each pathogen. George Ludwig (B. anthracis), David Hoover (B. meletensis), Luther Lindler (Y. pestis), Neill/Jett (SEs), and Yang (cholera toxin). The cell exposures were carried out using exposure concentrations/doses and other conditions that had been established in their laboratory. For in vitro exposures, the useful time frame ranged from 2-12 hours. In vivo, the time periods reflected the time course of the progression of illness, but blood samples were drawn prior to onset of illness, since gene patterns would be expected to precede the display of illness.

RESULTS AND DISCUSSION

Our laboratories have been carrying out gene expression profiling of the host response to numerous infectious and biological threat agents, however this manuscript will be limited to

discussions of studies with anthrax, SEs, LPS, plague, Brucella and cholera toxin. The initial screening used commercial gene arrays studying the host immune response *in vitro* using elutriated human peripheral blood mononuclear cells (PBMC). We have also carried out experiments to compare in vitro and in vivo results by utilizing PBMC obtained from non-human primates challenged with the specific biological threat agent.

Our initial work with global gene analysis studies was directed to determine the extent of the similarities and differences in PBMC responses in vitro to two classical shock-inducing toxins, staphylococcal enterotoxin B vs lipopolysaccharide (LPS), the smallest active unit of endotoxin (5, 9, 18). For these two toxins, the progression of illness is quite similar but we know from the volumes of studies on each toxin that there are some specific differences in production of mediators throughout the course of the illness. Indeed we found certain common patterns in gene expression profiles, especially for sets of genes relating to production of subgroups of inflammatory mediators and their accessory molecules, but the confluence of responses showed high correlation between the two toxins for genes relating to the common eventual lesions, such as pulmonary distress and loss of regulation of vascular tone. Figure 1 shows an example of SEB or LPS-induced changes (relative to controls) in expression of a gene that codes for a protein regulator of vascular tone. It is an example of genes showing similar responses to both toxins and this expression pattern also was observed in monkeys challenged with SEB. It is essential to carry out these experiments observing gene alterations at increasing exposure time periods since many genes show time-dependent expression patterns. The kinetic changes in gene expression are especially critical in vivo. An example is that genes coding for cytokines usually display up-regulation at early time periods and the expression levels frequently disappear as time progresses. That is not an unexpected finding, since cytokine production can be seen for brief periods of time following exposure to toxins (5, 9, 16-19).

In contrast to the similarities in gene expression just described, clear differences were seen in response to SEB vs LPS for expression levels of genes coding for numerous cytokines and their accessory molecules, many of the signaling cascade molecules and a variety of other surface adhesion molecules, etc. This probably represents the differences in initial cellular receptors and their linked signal transmission cascades. This study pointed out to us that gene expression changes could show differences in patterns for each agent, but also show similarities relating to common eventual lesions, such as loss of regulation of vascular tone, the hallmark of lethal shock. The unique and common patterns of gene expression were confirmed in SEB-challenged monkeys. Of course, these studies obviously identify new therapeutic intervention sites and, furthermore, predict the time period during which that approach could be usefully targeted. This information provided the foundation for the current study of multiple biological threat agents, since identification of course of impending illness (such as loss of regulation of vascular tone, vascular leakage, pulmonary or renal distress, etc) could provide key information should there be exposures to unidentifiable agents. Gene expression responses occur prior to production of their corresponding proteins, and there is frequently a time lag for the concerted action of the causative proteins to result in the demonstration of the lesion. Therefore, gene expression studies offer an early glimpse into the course of the impending illness and shows in a time-dependent manner when a specific therapy regimen might be effective.

Creation of a library of gene expression responses to biological threat agents. The studies with SEB vs LPS showed that unique gene patterns resulted from exposure to each toxin, but common genes, very few of which had previously been described for these toxins, showed associations predictive of the eventual lesions known to be induced by both toxins. Therefore, we carried out experiments, first in vitro in human PBMC, to determine the pattern of gene expression in response to exposures to B. anthracis, Y. pestis, B. meletensis, SEB and cholera toxin (CT). The latter toxin was chosen essentially to aid in interpretation of the data and for comparisons with the other biological threat agents, since a wealth of information exists about biochemical pathways and their relationship to lesions for CT. For the studies with these 5 pathogens listed above, we accumulated the gene expression response patterns and began to mine from the data the examples of pathogen-specific changes in host immune response gene profiles and identified unique genes that could potentially be used as diagnostic markers and also serve as therapeutic targets. The data amassed in this study are voluminous. Figure 2 is a condensation of results that shows patterns of gene alterations common to more than one agent and other changes that are unique to a particular agent. In this table, red/pink indicates up-regulated genes while green/blue identifies down-regulated genes. This is but a small set of gene expression changes and they are sorted according to the listed functional activities. In this table, there are numerous examples of specific genes that respond to one of these toxic agents, while there are other examples of genes that are altered in response to most or all of the pathogens (see interleukins). As a group, genes coding for cytokines/chemokines were altered by multiple biological threat agents, although specific mediator receptors frequently showed individual responses. In Figure 2, time dependency is shown for all of the pathogenic agents except cholera toxin). The kinetics of gene expression responses is especially important, as pointed out previously, since it reveals potential therapeutic targets that may provide effective intervention at specific time periods during late stages of illness.

NHP-challenge: B. anthracis. We have verified numerous changes in gene expression in non-human primates exposed to B. anthracis at T=0, 24, 48, 72 h post-exposure. We again found, in general, that cytokines appeared primarily at T=24 h while genes involved in apoptosis and cell death were up-regulated at 48-72 h. Many unanticipated changes in gene response have provided potential therapeutic targets for late treatment.

SUMMARY

We found a series of gene alterations in PBMC from monkeys challenged with SEB that confirm the gene pattern observed in vitro. In cells from the challenged monkeys, we have defined a series of genes, the expression of which was altered in the pattern typical of SEB, not LPS or other shock-inducing toxin by 30 min post exposure. Since onset of illness begins at approximately 3 h post exposure for SEB, early detection of the gene pattern could permit initiation of appropriate countermeasures prior to, or at least in the early stages of illness. In the case of B. anthracis, by 48-72 h, gene patterns from challenged monkeys show resemblances of the "lethal shock" profile that was seen at early time periods with SEB. Indeed, anthrax infection eventually proceeds to lethal shock. One of the purposes of relating gene alterations with a course of illness is that exposure to unknown or genetically altered agents could be "categorized" as to expected lesions and can provide the opportunity to initiate preventive therapy prior to onset of severe illness.

ACKNOWLEDGEMENTS

This work was supported initially by seed funding from an In-house Investigative Laboratory Research (ILIR) grant, and supplemented with funds from USAMRMC, RAD IV, Common Diagnostic Systems. The majority of the funding for the study was obtained from Defense Advanced Research Projects Agency (DARPA), competitive grant award number H 960.

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FIGURE LEGENDS

Figure 1. Changes in Gene Expression in Response to Two Shock-Inducing Toxins. Gene coding for a protein involved in regulating vascular tone shows the example of similarities that result from exposure of PBMC to SEB or LPS in vitro. Expression of this gene was also down-regulated in monkeys challenged with SEB. Although for this particular gene, the kinetics of gene expression was similar over the 24 h time period, that is frequently not the case. Therefore, study of gene responses in a time-dependent manner is essential, especially for in vivo studies, in order to understand the useful time frame for intervention aimed at potential therapeutic targets.

Figure 2. Gene Array Analysis of PBMC Treated with Biological Threat Agents. Each agent was studied at different time periods, usually 2,4,8 hr and for SEB, an additional time period of 18 h. CT was studied at 16 h. Red/pink indicates up-regulated genes while green/blue shows down-regulated genes relative to control samples. The kinetics of gene responses for each different agent provide important information for possible late-stage therapeutic targets. Some gene classes show responses with most of these pathogenic agents (interleukins) while a host of other genes show patterns that could distinguish one pathogenic agent from another.

Changes in Gene Expression in Response to Two Shock-Inducing Toxins.

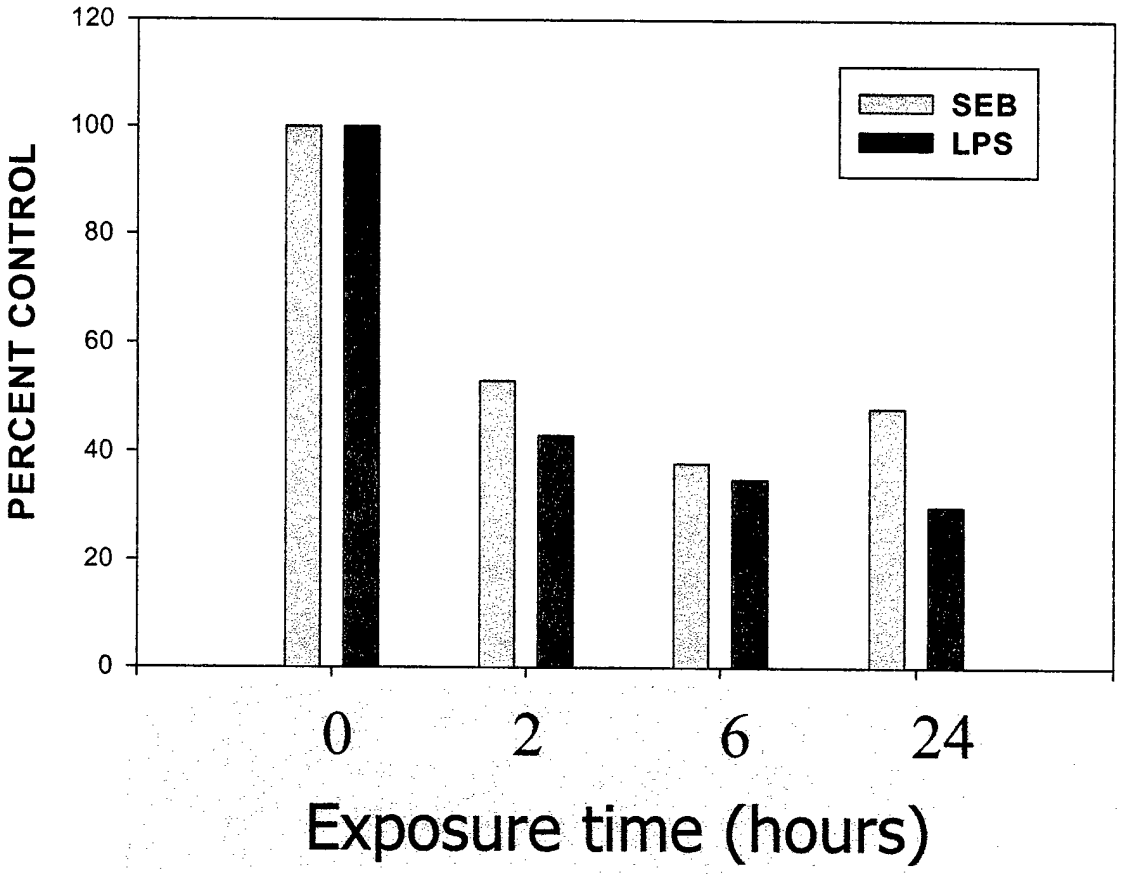
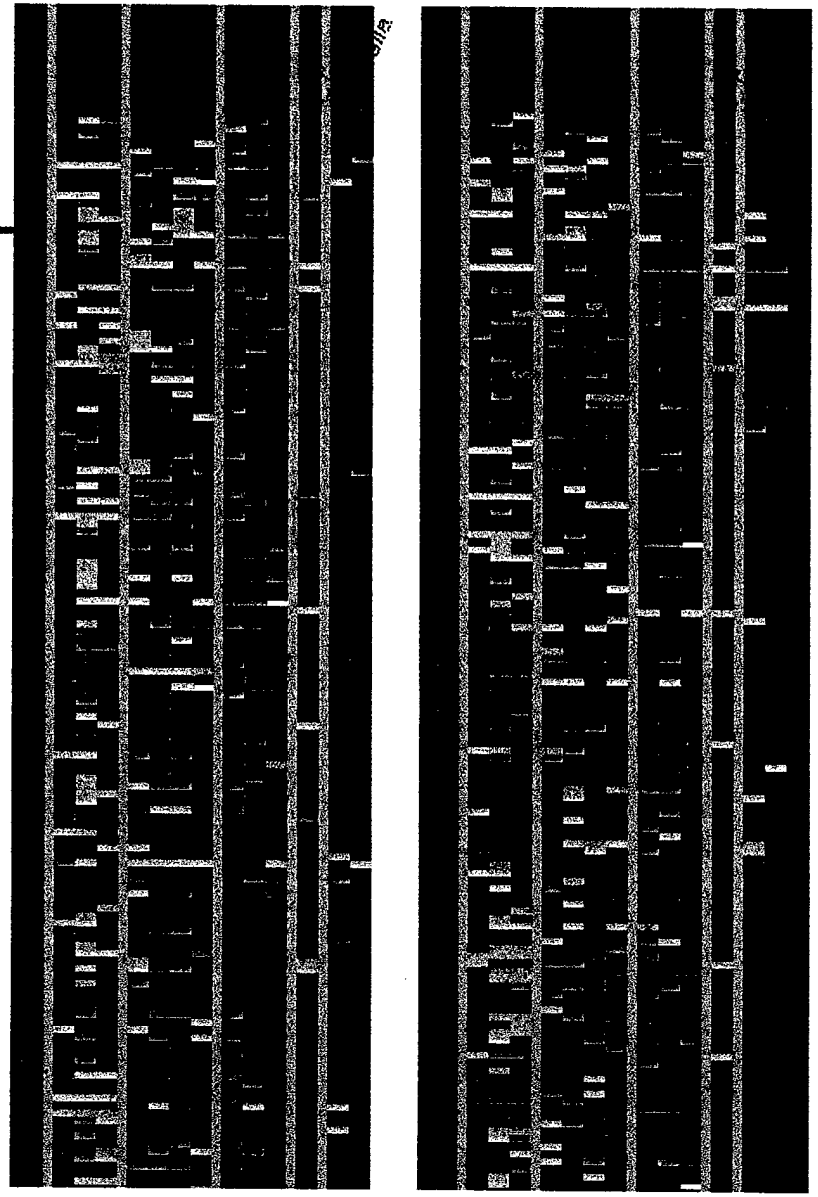


FIGURE 2



Cyclases

Calpains

Caspases

Cell cycle

Adhesion molecules

Cyclins

Death Assoc

DNA Damage ligases

Cytokines & Chemokines

Homeostasis & detoxification enz

Hormones & receptors

Inflammatory mediators

Interferons & Receptor

Interleukins & recep

Intracellular kinases