

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE Oct 1998 | 3. REPORT TYPE AND DATES COVERED Final, Proceedings, Oct 1998 | |
| 4. TITLE AND SUBTITLE ALTERATIONS IN GENE EXPRESSION SHOW UNIQUE PATTERNS IN RESPONSE TO TOXIC AGENTS 21st Army Science Conference, Proceedings 21:529-534 | | 5. FUNDING NUMBERS ILIR | |
| 6. AUTHOR(S) Rina Das, Chanaka Mendis, Zhengyin Yan, Roger Neill, Thomas Boyle and Marti Jett. | | | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Division of Pathology Walter Reed Army Inst. Res. 503 Rotert Grant Road Silver Spring, MD 20910 | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) 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 <b style="font-size: 2em;">20020613 047 | |
| 13. ABSTRACT (Maximum 200 words) We have previously observed that various toxins produced a distinctive pattern in production of mediators of illness when using either cultures of human lymphoid cells or when using plasma and/or lymphoid cells from animal experiments. We predicted that each toxin would alter gene expression in a unique pattern. With staphylococcal enterotoxin B as a prototype, we have identified 15 genes that were altered using the powerful technique of differential display. Our preliminary data using other toxins suggests that each induces a unique pattern of alteration in gene expression. Lymphoid cells provide a readily accessible reservoir of information that can reveal direct or indirect responses to toxic agents. These data indicate the potential usefulness of establishing a library of gene expression alterations in response to a battery of threat toxic agents. This will provide a means of determining a) the probable course of impending illness if structural-based toxin identification was unsuccessful and b) the presence of priming contaminants, which would make the illness more acute. Additionally, the pattern of expressed genes can lead to assessment of the expected course of illness and initiation of treatment modalities prior to onset of symptoms. | | | |
| 14. SUBJECT TERMS Biological warfare agents, Host responses, gene expression pattern, Anthrax, Brucella, Plague, Dengue, Staphylococcal enterotoxins, SEB, lipopolysaccharide, cholera toxin, Diagnostic marker genes, lymphoid cells, potentiating agents, unidentified pathogens. | | 15. NUMBER OF PAGES 6 | 16. PRICE CODE |
| 17. SECURITY CLASSIFICATION OF REPORT unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT unclassified | 20. LIMITATION OF ABSTRACT unlimited |

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ALTERATIONS IN GENE EXPRESSION SHOW UNIQUE PATTERNS IN RESPONSE TO TOXIC AGENTS

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Abstract

We had previously **observed** that various toxins produced a distinctive pattern in production of mediators of illness when using either cultures of human lymphoid cells or when using plasma and/or lymphoid cells from animal experiments. We predicted that each toxin would alter gene expression in a unique pattern. With staphylococcal enterotoxin B as a prototype, we have identified 15 genes that were altered using the powerful technique of differential display. Our preliminary data using other toxins suggests that each induces a unique pattern of alteration in gene expression. Lymphoid cells provide a readily accessible reservoir of information that can reveal direct or indirect responses to toxic agents. These data indicate the potential usefulness of establishing a library of gene expression alterations in response to a battery of threat toxic agents. This will provide a means of determining a) the probable course of impending illness if structural-based toxin identification was **unsuccessful** and b) the presence of priming contaminants, which would make the illness more acute. Additionally, the pattern of expressed genes can lead to assessment of the expected course of illness and initiation of treatment modalities prior to onset of symptoms.

1. Introduction

The increased sophistication available for design of potential biological weapons will require reliance on various approaches to adequately identify such threats. Simple identification of toxins or infectious agents, using a battery of structural-based probes, may be complicated by the fact that genetic manipulations can make these agents unidentifiable by standard techniques and can, concomitantly, enhance their devastating effects. Furthermore, small amounts of common bacterial products, such as protein A or endotoxin, have been shown to markedly potentiate activities of biological warfare threat toxins. Therefore, our laboratory has pursued a different approach, which is to define the response to a toxic agent (possibly unidentified) by comparison to a library of known responses to toxins. Specifically, our overall goal is to use our findings of lymphoid cell responses to toxic agents to establish a library so that treatment can be initiated quickly and can be designed as a countermeasure appropriate for the agent(s) involved. For this approach, confirmed identification of a biological warfare agent is not necessary, nor is it essential to establish if minute quantities (perhaps at undetectable, but biologically active levels) of potentiating bacterial products contaminate the biological weapon (BW) used.

As **prototype toxic agents** in our initial studies, we have assessed the biologic effects on lymphoid cells by certain toxins that induce lethal systemic shock in primates. Though different mechanisms, both endotoxin (lipopolysaccharide [LPS] of gram-negative bacteria) and staphylococcal enterotoxin B (SEB) induce production of a cascade mediators whose activities lead to shock. The release of LPS from the cell wall of gram-negative bacteria, and subsequent production of numerous host mediators, is the initiating event of septic shock (Pugin, 1993; Wright, 1990). In contrast, SEB acts as a **superantigen**, stimulating T cell proliferation (Jett, 1994; Neill 1996), inducing a number of cytokine genes and other mediators in lymphocytes and monocytes (Yan, 1997a). In our laboratory we have shown that SEB induces high levels of CD69 (Yan, 1997b) while LPS produces a minor change in this surface marker. In contrast, TNF- α production is rapidly elevated by LPS whereas SEB produces modest changes in its production (Yan, 1997a,b). These changes which we have characterized are just two of a battery of potential biomarkers indicative of patterns of impending illness. Production of a unique pattern of mediators of serious illness in response to toxic agents, we predict, will be indicative of the type of illness or toxicity that will follow. We have now proceeded to identify not just the mediators, but also the genes altered in response to toxins +/- LPS using the technique of differential display.

The differential display approach was introduced in the past few years and has become a potent tool for identifying genes that are differentially expressed in various eukaryotic cells and organs or under altered conditions. This technique is highly sensitive and reproducible, and is a rapid method for identifying unique genes, quantitatively, which are altered upon treatment of cells with the compound of interest. Not only will this information provide a library of genes that are activated by toxins/agents producing serious illness, it will aid in identification of new treatment modalities. Thus this technique has

enormous potential; identifying the changes occurring at the molecular level in a system has radically changed concepts in biomedical research by opening new avenues for diagnosis and therapy.

2. Materials and Methods

2.1 Primary Cell Cultures: Cell isolation and purification from plasma of healthy human donors.

Human lymphocytes and monocytes were prepared from leukopacks from normal donors according to Jett et al 1994 using lymphocyte separation medium **histopaque** 1077. Lymphocytes and monocytes were purified and separated further by counterflow centrifugation-elutriation with PBS as the eluant.

2.2 ELISA Measurement of Mediators.

In vitro cultured cells and their culture fluid or plasma/ lymphoid cells isolated from animals treated with SEB were used to measure production of mediators of illness such as cytokines, eicosanoids, etc. All samples were frozen as aliquots (to avoid freeze/thaw cycles) at -70°C immediately upon collection. In assays for eicosanoids, plasma extractions were necessary. They were performed exactly as described by Boyle et al 1994. In each case, the manufacturer's instructions were followed. **TNF- α** and **IL-6 reagents** were obtained from the Intergen Corp. (Purchase, NY); fluorescent reagents to quantitate eicosanoids were obtained from **PerSeptive Biosystems** (MA); CD-69 detection and its correlation with CD-3 and CD-14 was accomplished using fluorescent antisera (**Becton-Dickenson**, CA) which recognized each surface marker. Antisera were added 20 h after addition of toxin. Cholera Toxin was used at the concentration of 5nM.

2.3 Signal Transduction: Treatment and extraction

To understand the molecular events induced by SEB and LPS in human lymphocytes, we treated them with SEB and or LPS for various periods of time (0S-30 minutes) and assayed for the activity of several signaling kinases to identify the pathway for signal transduction. The JNK, p-38 were tested for their activation upon SEB treatment.

Human **lymphocytes/monocytes** were mixed in 4:1 ratio at a final density of 2.5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% human AB serum. Cells were treated with 25-100ng/ml of SEB and 50-100ng/ml of LPS for different time periods. The cells were lysed after brief sorication and incubation in the lysis buffer containing 20 mM HEPES, 10 mM EGTA, 40 mM β -glycerophosphate, 2.5 mM MgCl_2 , 1 mM DTT, 150 mM NaCl, 2 mM Sodium Orthovanadate, 1 mM PMSF, 10 $\mu\text{g/ml}$ Leupeptin, 10 $\mu\text{g/ml}$ Aprotinin, 1% NP40 and 0.5% Deoxycholate.

Equal amounts of protein were resolved on a 10% SDS-gel and transferred to a nitrocellulose filter paper. The western blots were probed with a p-JNK specific antibody that specifically recognises the active form of the enzyme and detected using anti-HRP conjugated secondary antibody and ECL detection kit (Amersham, Downers Grove, IL).

For immunoblots cell lysates were immunoprecipitated with a specific antibody and protein-G Sepharose at 4°C for overnight. The precipitate was washed twice in lysis buffer and resolved on a gel.

2.4 Differential Display:

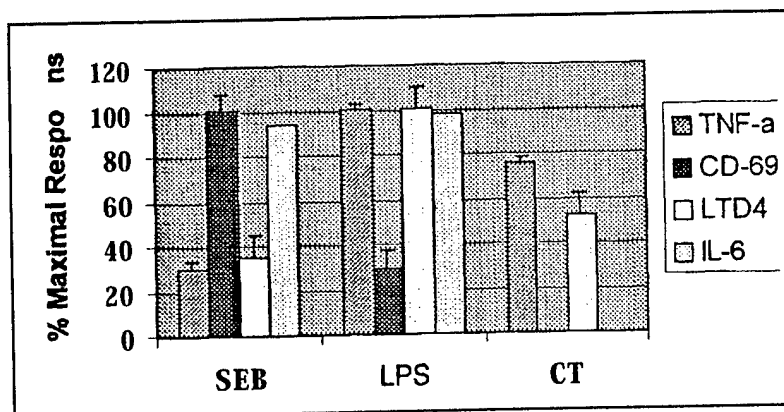
For the Differential Display PCR (DD-PCR) technique, human lymphocytes, which are very responsive to SEB were used. We treated 4:1 mixture of Lymphocytes: Monocytes with 100 ng/ml of SEB or 50 ng/ml of LPS for different time periods (2hr-72hrs), isolated the total RNA by the Trizol method and proceeded with the DD-PCR. For the reverse transcriptase reaction we used different anchored primers (AP) with dT11. PCR was performed in duplicates to avoid false positives. We used a set of arbitrary primers (ARP) with the same anchored primers used in the RT reaction along with α -³²P ATP as one of the nucleotides. The anchored and the arbitrary primers were obtained as a kit from Genomx Corp, CA. The PCR products were resolved on a 4.6%-6.4% Long Read Gel run in a LR-Sequencing gel apparatus also obtained from Genomx Corp, CA. After the gels were washed and dried, they were exposed to X-ray films and the bands were compared for differences. Relevant bands with at least 5-6 fold differences were excised from the gel and reamplified using the M13 and T7 primers. Using two different anchored primers and 4 different arbitrary primers 15 different bands have been isolated. These genes were sequenced by Cycle Sequencing Ki (Amersham) and have been compared with the GENBANK or EMBL databases to identify the genes.

3. Results

3.1 Changes in levels of various mediators of illness in response to selected toxins.

We have studied the toxin-induced release of mediators in cultures of primary human lymphoid cells. The patterns of mediators released in response to 3 toxins are shown in Figure 1. Human lymphoid cells were incubated with SEB, CT or LPS overnight and production of TNF- α and IL-6 were measured by ELISA; CD-69 positive cells were determined by

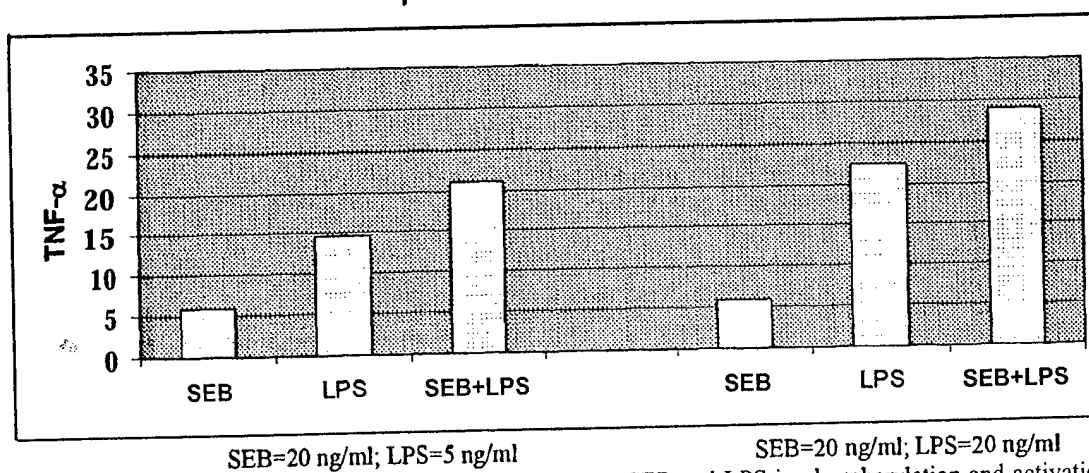
Figure 1. Mediators of illness produced in response to various toxins in cultures of human lymphoid cells. Data are average of results obtained with cells from three different donors.



flow cytometry. LTD4 was determined by immunofluorescence 2 min after addition of the toxins. The overall impression of the pattern of mediator release indicates that the related toxins, SEB and TSST-1 (data not shown), display a similar pattern in mediators produced. In contrast, the pattern of mediators produced upon LPS stimulation is markedly different from the staphylococcal enterotoxins and cholera toxin. Mediators most elevated in response to LPS were **TNF- α** and **LTD4**; Cholera toxin and SEB each showed a mid-level induction of the appearance of LTD4, which was about half that seen with LPS. There was approximately a 3-fold greater increase in CD-69 positive cells upon exposure to SEB vs LPS. IL-6 production, although greatly elevated above control values, was very similar for both SEB and LPS. IL-6, therefore, would possibly not be useful in discriminating among toxins, although it could provide information confirming predicted course of illness. These fold-changes in mediator levels, are reproducible and do not vary appreciably among the approximately 20 leukaphoresis (healthy) donors who have participated in our protocol over the years these studies have been performed.

Small amounts of common bacterial products, such as protein A or endotoxin, have been shown to markedly potentiate activities of staphylococcal enterotoxins (Henne, et al, 1991). Therefore, we examined their effects on lymphoid cells both separately and together. Human lymphoid cells were treated with SEB or LPS overnight and production of **TNF- α** was measured by ELISA. LPS stimulated release of significantly more **TNF- α** than SEB, however when both toxins were present at very low concentrations there was an additive response of **TNF- α** production (Fig. 2).

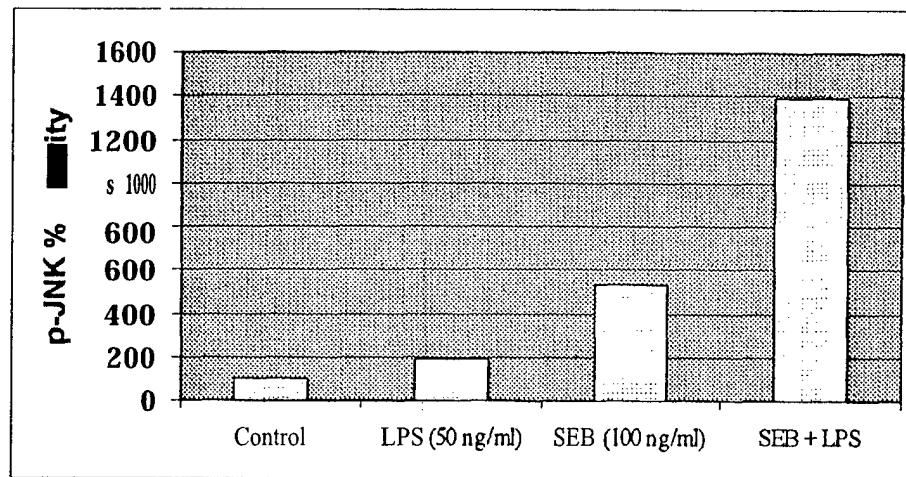
Figure 2. **TNF- α** production in response to SEB and /or lipopolysaccharide in human lymphoid cells.



Using p-JNK as the endpoint, we observed synergy between SEB and LPS in phosphorylation and activation of stress-induced kinase. Stress activated p-JNK is a signaling kinase activated by *W*irradiation, arachidonic acid,

actinomycin D and other stress inducing agents. When small amounts of LPS (50 ng/ml) were added to the SEB (100 ng/ml) treated cells, we observed a synergistic effect on phosphorylation. In the presence of SEB there was a five-fold activation of JNK compared to the control, however in the presence of LPS and SEB the activation was 15 fold (Fig 3). Presence of LPS enhanced the SEB effect significantly on the activation of these stress activated protein kinases.

Figure 3. Synergistic effects of SEB and LPS on p-JNK activity in human lymphoid cells



3.2 Changes in Gene expression induced by SEB.

We have observed striking differences among toxins in mediator production *in vitro* using primary cultures of lymphoid cells. Furthermore, the mediator responses we have observed in our studies with toxins have been consistent despite the fact that many different people are in our donor pool for obtaining the cells. In addition, we have confirmed that similar patterns of mediators are produced in non-human primates challenged with SEB. However, mediators are unstable, extensive sample preparation is required and mediators are labor intensive to measure. Therefore, we decided to examine the changes in levels of gene expression induced by these toxins. Using SEB as a prototype, we studied changes in expression of mRNA using selected RT-PCR primers and subsequently performed the technique, differential display (DD).

For observing changes in gene expression patterns, the human lymphoid cells were treated with SEB for various time periods and, after DD-PCR reactions, we have identified changes in expression patterns of 5 genes (9 up- and 6 down-regulated). To this date, the 9 shown in Table 1, have been isolated, cloned, sequenced and characterized. The

Table 1. Changes in Gene Expression Identified in Lymphoid Cells Treated with SEB*

| GENE | PRIMERS | CHANGES IN EXPRESSION | FUNCTION |
|------|--------------------|-----------------------|--|
| | Anchored Arbitrary | | |
| #1 | AP3 ARP3 | DOWN REGULATED | Involved in early stages of wound healing; has heparanase-like enzymatic activity. Binds to proteoglycans. |
| #2 | AP3 ARP4 | DOWN REGULATED | A member of the class of proteoglycans involved as adhesion molecules and regulators of cell proliferation. |
| #3 | AP1 ARP2 | UP REGULATED | A NOVEL GENE. No matching sequence have been found in either GENBANK and EMBL databases. |
| #4 | AP1 ARP2 | UP REGULATED | IL-6. A cytokine involved in inflammation, T-cell proliferation and release of cascades of other mediators |
| #5 | AP3 ARP3 | UP REGULATED | A contractile protein which had been characterized as contributing to cardiomyopathy; A regulator of motor activity. |
| #6 | AP3 ARP3 | DOWN REGULATED | Currently no positive match with gene database sequences |
| #7 | AP3 ARP4 | DOWN REGULATED | Currently no positive match with gene database sequences |
| #8 | AP1 ARP2 | UP REGULATED | Currently no positive match with gene database sequences |
| #9 | AP3 ARP2 | UP REGULATED | Currently no positive match with gene database sequences |

* Cells were exposed to SEB at 100ng/ml.

remaining 6 are currently being sequenced and characterized. Note that the initial product was mRNA which was isolated from the SEB-exposed cells; it was reverse transcribed to obtain the gene for that mRNA (the GENBANK and EMBL databases contain only sequences of DNA). The procedure used was that after identification of the changes, the bands were excised and the genes were reamplified using M13 and T7 primers. The genes were sequenced using Cycle sequencing reagents from Amersham and the results are summarized in Table 1. Nine genes have now been sequenced. Four gene sequences were identified by matching the sequence to those of known genes that are catalogued in the GENBANK and EMBL databases. Those four genes are described by function in Table 1. Gene #4 was elevated 5-fold over controls by SEB as determined in these differential display experiments; it has been identified from database comparisons as the gene for IL-6. The mediator protein IL-6 is well documented by us and others as being elevated in response to SEB and other toxins. We expected that we should find elevated cytokine message in these studies with SEB stimulation of lymphoid cells. Identification of the 8-fold increased expression of mRNA for IL-6 illustrates the correlation of these experiments with known information about SEB action. Genes 1, 2 and 5 have been positively identified by database comparisons. These are genes coding for proteins, not previously implicated in SEB action on lymphoid cells. They have varying activities and functions; there is a common theme of association with adhesion molecule function. These proteins may provide clues for new approaches in the treatment of lethal shock.

We have identified one gene (#3), which is up-regulated in response to SEB exposure that does not match any known gene sequence; we have used every available search technique and database and, therefore, conclude that this is a novel gene that has not been identified previously. Four other genes (gene #'s 6-9) have only partial matches to database genes. The same search methodology is being performed with these genes to determine if they are newly identified genes.

4. Conclusions

These studies show that toxins have unique patterns in production of mediators associated with illness (Fig. 1). Furthermore, our studies have shown elevation of not only the mediator, itself, but also in expression of corresponding genes for both IL-6 (Table 1) and TNF- α (data not shown). Several genes, the expression of which is altered by SEB (Table 1), have at least ancillary functions as adhesion molecules (genes 1, 2, and 5). The down-regulation of the gene #1, involved in wound healing, is intriguing in view of our findings that SEB disrupted barrier functions in pulmonary artery endothelial cells (Campbell, et al 1997). These genes may provide new targets for treatment of lethal shock. Preliminary studies using other toxins indicate that differences in gene expression alterations show a unique pattern upon exposure to a BW agent. Peripheral blood lymphoid cells have been selected for use in these studies since they can be readily obtained from an exposed individual and serve as a reservoir of information. Indeed, some toxic agents will have lesser effects on lymphoid cells than do SEBs, but it is likely that some gene alteration will occur in lymphoid cells, directly or indirectly, in response to most, if not all BW agents and even infectious agents.

Toxicity enhancers: Since LPS contamination of BW agents would be expected to be unavoidable, if not deliberate, the vast potentiation of other toxic agents by LPS introduces complexities that need to be considered. In the case of the SEBs, the incredible potentiation by LPS is well documented in the literature (Henne, et al, 1991). LPS has a priming effect, which can enhance sensitivity to a variety of toxins; it markedly increases SEB-induced lethal shock. We questioned whether or not responses to either toxin, alone, would be additive or synergistic when they were administered together, we found examples of each. In the case of TNF- α , the two toxins, together, produced additive levels of the mediator (Figure 2). This may not be surprising since we (Yan, et al, 1997a,b) have found that SEB stimulated production of TNF- α from T-lymphocytes and not from monocytes, while LPS did the opposite, stimulating TNF- α production from monocytes and not lymphocytes. Each cell type was maximally affected by the appropriate toxin and would not respond further.

Synergy between SEB and LPS was seen upon examination of stress-activated signaling cascades, phosphorylation of p-JNK (Figure 3). This is an early event triggered by SEB and LPS. Signal transduction pathways and genes that are activated by these agents are not understood very well. The stimulation of this stress-induced kinase was much more pronounced in the lymphocytes when compared with the monocytes alone. In the presence of small amounts of both LPS and SEB, activation of JNK was increased dramatically when compared to either SEB or LPS alone.

Gene expression patterns: In the course of our studies with SEB, we have observed increased expression and activation of several critical genes that we expected to be involved toxin-induced lethal shock (such as 5-lipoxygenase, various cytokines, etc). However, we recognized that it was important to identify an overall battery of genes that are altered in response to these biological toxins. This presents a more complete picture of the toxin action and provides information that may be exploited for assessment of exposure and initiating early treatment. It was for these reasons that we examined gene expression in response to toxins using the technique of differential display.

We propose to extend the gene screening process to other toxins as well. This will create a library of gene responses to BW agents; we expect that these agents will fall into groups causing similar gene alterations. These genes will act as markers, in a time-dependent manner, predicting the pattern of illnesses before the actual symptoms appear.

Identification of specific genes that are differentially **expressed** in response to BW agents will give a better idea of their **specific** targets. The identified genes and their function will be studied in detail, which will enable us to block gene(s) which lead to impending severe illness. The molecular changes in the lymphoid cells with these prototype toxins can be eventually extended to other toxins and/or infectious agents that cause serious illness in humans.

This approach has enormous potential for providing critical information in the event of BW exposure. Various commercial products [Genetics Institute (Cambridge, MA); Genomix (Palo Alto, CA)] exist in which primer gene sequence arrays can be placed on a matrix "chip" that can simultaneously screen up to 500 genes at one time. Clearly the approach we have taken lends itself to that type of **technology**. We have already found **selected** genes, the expression of which is altered by SEB, but not by LPS. This futuristic approach requires that we identify and select a pattern of unique genes for a battery of threat toxins. The system can be readily verified in animal models challenged with the threat toxin. The crucial factor is the establishment of the **library** of responses of lymphoid cells to a **battery** of threat toxins and/or agents. Development of new gene screening technologies will, likely, exceed even current expectations.

In summary, at the present time we have identified 15 genes with altered expression, which have been observed upon SEB exposure to peripheral blood human lymphoid cells. These cells can be obtained readily from patients and provide a reservoir of information due to their responses to toxins, infectious agents, etc. We have **catalogued** patterns of responses for several toxins; the objective would be to relate genes expressed in response to a biological warfare insult, to a map of responses predictive of physiological responses. One **need** not know the identity of the toxic agent to determine the likely progression of symptoms, based on markers/mediators: induced. The advantages in screening for **specific mRNA** induced by BW agents is that it will provide a target for early detection of surrogate markers of impending illness. Having identified what genes are effected by the toxins, we will be able to design strategies for treatment approaches to block their function and thus prevent the lethal shock.

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