Applications and Challenges of DNA Microarray Technology in Military Medical Research

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This review discusses the challenges and applications of DNA microarray technology as it is being used in each of the four major research areas of the U.S. Army Medical Research and Materiel Command: military infectious diseases, combat casualty care, military operational medicine, and medical chemical and biological defense. The overall objective of this review is two-fold. First, the objective is to increase awareness in senior military leadership of the challenges and opportunities presented by DNA microarray technology and the emerging and rapidly changing field of bioinformatics. Second, the aim is to publicize to the civilian research community the additional challenges associated with the use of microarray technology in military medical research. This discussion contains material that would be useful for making programmatic recommendations that team strategic research investments and emerging technologies with U.S. Army Medical Research and Materiel Command resources.

Introduction

Deoxyribonucleic acid microarray technology[1-5] is being exploited in a number of projects within the U.S. Army Medical Research and Materiel Command (USAMRMC). These projects are drawing a growing number of investigators from the four research focus areas of the Command: military infectious diseases, combat casualty care, military operational medicine, and medical chemical and biological defense.[6] Unlike older assays, DNA microarrays offer the possibility to simultaneously interrogate tens of thousands of genes and can offer a genome-wide snapshot of gene expression levels in a given condition. (An in-depth description of the DNA microarray technology and its associated analysis challenges can be found in Schena[7] and Draghici[8], respectively).

The specific goals of this review are to provide an overview of the USAMRMC's main research areas and a sample of related projects using DNA microarray technology and to identify current challenges related to the use of DNA microarray technology that are prevalent, if not unique, in military medical research. These should lead to programmatic recommendations that team strategic research investments and emerging technologies with USAMRMC resources. The intended target audience of this article ranges from senior military leaders knowledgeable of the command's research mission but not skilled in DNA microarrays, to civilian researchers proficient in this new technology but unaware of military medical research aims and unique requirements.

It is recognized that some of the DNA microarray issues identified here are also germane to the civilian research community. However, the unique nature of the military medical research often results in unique needs and requirements that exceed those in the civilian setting. For instance, toxicological applications of gene expression in the civilian world are focused on the drug development process (absorption, distribution, metabolism, elimination, and toxicity).[9,10] Environmental toxicology applications are only just beginning to be explored by the respective research community.[11-13] On the other hand, military research in the realm of toxicology extends such interests to the effects of chemical warfare (CW) agents (CWAs) and other toxic exposures, which poses additional requirements not generally experienced in civilian research. (After the recent terrorist attacks, certain medical issues once believed to relate strictly to the protection of military forces in war environments, such as the defense against CWAs and biological warfare agents (BWAs), now also relate to the public at large and homeland defense.)

In certain cases, the research needs and requirements are specific to particular military relevant missions. For example, the need to investigate postdeployment health concerns, such as Persian Gulf War illness, to identify biological markers to ascertain susceptibility of illnesses in humans exposed to extreme environmental conditions, and to investigate biomolecular responses to potential exposures to hazards and threats faced by soldiers may have no parallel in the civilian setting. In other cases, they are equally applicable to public health, but the "market" is not sufficiently large to justify resource investments by private pharmaceutical and medical device manufacturing companies. For instance, infectious diseases, such as leishmaniasis, hantavirus, and secretory diarrhea, have military and public health relevance. However, because these diseases are not currently prevalent in developed nations, research efforts are somewhat limited to the military. A recent historical example in which military research benefited the civilian sector is in malaria research. Currently, one of the prophylactics recommended by the Centers for Disease Control and Prevention is mefloquine HCl, which was developed as WR-142,490 under the aegis of a predecessor of USAMRMC and became the first "orphan drug" approved by the Food and Drug Administration.

After a brief description of the four research focus areas of the USAMRMC, the subsequent sections present the major applications of microarrays in each of these four areas and identify the unique challenges in applying DNA microarray technology in military medical research. Making DNA microarray technology information available to the research community at large pro-

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vides opportunities for input on research issues, avoids duplicating research efforts, and fosters collaborations. Furthermore, this review could provide valuable information for educating military leaders in the promising and quickly growing and changing field of bioinformatics, and for crafting future requests for proposals to help align the directions of the USAMRMC strategic research investment with new developments and emerging capabilities.

The Four Main Military Medical Research Areas

Below is a brief description of the four major research focus areas of the USAMRMC: military infectious diseases, combat casualty care, military operational medicine, and medical chemical and biological defense. Additional information can be obtained at the Command's Website.[6]

The Military Infectious Diseases research program focuses on prevention, diagnosis, and treatment of diseases (e.g., malaria, dengue, scrub typhus, Japanese encephalitis, and adenovirus) that can seriously hamper military mobilization, deployment, and effectiveness. Research is centered on infectious diseases endemic to areas around the world where U.S. troops could be deployed, with emphasis on the discovery and development of prophylactic and treatment drugs for infectious diseases; the development of vaccines against serious military diseases; and technologies for rapid identification of disease organisms and diagnosis of infections.

The Combat Casualty Care research program focuses on reducing morbidity and mortality of soldiers sustaining life-threatening injuries on the battlefield. Research efforts address products and methods that will reduce the number of battlefield deaths from hemorrhage; advanced, noninvasive physiologic sensors for detecting penetrating or blunt trauma wounding events and remote triage; and diagnostics to help the medic on the battlefield to determine which casualties require immediate resuscitation.

The Military Operational Medicine research efforts are focused on producing biomedical solutions to protect, sustain, and enhance soldier performance and health in the face of the entire spectrum of operational stressors (e.g., cognitive load, fatigue, sleep debt, fear, and anxiety) and environmental threats (e.g., heat stroke, hypothermia, and altitude sickness). Research emphasis includes physiological studies to improve soldier performance, adaptation, and survivability involving extreme environments, including heat and cold stress, and to optimize the medical management of associated diseases and injuries; the study of pharmacological, training, and nutritional intervention strategies to optimize performance, such as mitigating the effects of sleep deprivation and fatigue in sustained operations; and health effects of environmental toxic hazards, including occupational- and environmental-related chemical hazards of military uniqueness and relevancy.

The mission of the Medical Chemical and Biological Defense research program is to preserve combat effectiveness by timely provision of medical countermeasures in a CW or biological warfare (BW) environment. Accordingly, the research efforts address mechanisms of action by which CWAs/BWAs exert their toxic effects to develop and test prophylactic agents and therapeutic modalities; mechanisms of action by which antidotes interact with and induce their ameliorating effects of CW/BW injury or disease to enhance their efficacy and safety; vaccine technologies, drugs, and antisera for the prevention of disease caused by BWAs; improved diagnostic tests for rapid identification of CW/BW intoxication and/or illness; and antidotes, chemoprophylaxes, pretreatments, and therapeutic compounds for the prevention and treatment of the effects of CWAs.

In each of these four research areas, USAMRMC investigators are exploiting DNA microarray technology to examine global cellular and tissue response to toxic chemicals, infectious agents, disease, inflammatory and physical insults, and pharmaceuticals to elucidate mechanisms of action and how potential therapies may intervene at a molecular level. A representative cross-section of current studies exemplifying these applications of microarrays is presented below.

Military Medical Applications

Military Infectious Diseases

Current DNA microarray efforts in military infectious diseases are centered on studies that can help in the early diagnosis of infection, in the understanding of the pathways of infection and the molecular mechanisms of resistance to drugs, and in the elucidation of the mechanisms that cause different manifestation of disease and protection, leading to the development of vaccines and drugs of improved efficacy. Endemic infectious diseases of military relevance that are currently investigated with gene expression array technology include dengue, malaria, and certain strains of human immunodeficiency virus (HIV).

In dengue infection, the use of gene expression microarray technology is being investigated in two parallel studies at the USAMRMC laboratories. In the first study, the aim is to develop a sensitive, rapidly deployable system for early diagnosis of dengue infection and prediction of disease severity levels.[14] This effort is directed toward the characterization of the cellular host response to exposure using human peripheral blood mononuclear cells (PBMCs) as an in vitro model system. The hypothesis is that PBMCs can be used as a readily accessible reservoir of historical information of what has transpired in the host, providing unique gene profile signatures capable of differentiating exposure types and predicting disease severity levels.

The second study is aimed at the identification of correlates of immunity in dengue-vaccinated volunteers challenged with near wild-type virus, ultimately leading to the development of more effective dengue vaccines and improved treatment of life-threatening dengue hemorrhagic fever. In this study, volunteers are vaccinated with the tetravalent (dengue 1–4) vaccine and are then exposed (along with nonvaccinated volunteers) to a near wild-type version of the dengue virus. Measuring the changes in protein expression in the subjects' plasma and correlating such changes with their immune response and clinical parameters allows for initial characterization of the different manifestations of the disease and helps us gain a better understanding of the host-protective mechanisms. To the best of our knowledge, the Army is pioneering the use of DNA microarray technology in dengue studies, as there are no such studies being performed in the civilian sector.

Research efforts related to malaria focus, among other things, on the issue of drug resistance. For instance, chloroquine is one of the most effective antimalarial treatments, but resistance to it

is becoming widespread. *Saccharomyces cerevisiae* was used as a model eukaryotic system for studying this phenomenon and improving the understanding of the molecular mechanisms of the resistance to drugs. This was done using an adapted Affymetrix yeast chip.[15,16]

A similar strategy is used in HIV-related research. Studies have used microarrays to profile the expression of the genes of human PBMCs infected in vitro with HIV. The goals of such research are to find the cellular functions impacted by viral infection and patterns of coordinated gene expression that might help elucidate fundamental cellular processes affected by the virus. Such knowledge can help in the understanding of the cellular mechanisms used by the virus and ultimately help develop ways of blocking its activity.[17]

Combat Casualty Care

Hemorrhage is the single greatest cause of morbidity and mortality in conventional warfare.[18] At the same time, 30 to 40% of civilian injuries involve hemorrhage, making it the leading cause of death for those less than 44 years of age in the United States.[19] The major working hypothesis in this research is that hemorrhagic shock—as well as other types of trauma-induced shock, such as burn—stimulates the inflammatory response of yet undetermined metabolic mechanisms as it attempts to restore homeostasis. The inflammatory response is known to be a transcriptionally mediated response and, hence, can be studied by monitoring changes in gene expression using microarrays.

Current research involves the determination of the tissues and genes most affected by hemorrhage with the goals of devising resuscitation strategies and identifying targets for therapeutic intervention based on this understanding. The hypothesis is that this information could be used to develop polydrugs, such as supplements for resuscitation fluids, which would inhibit or reduce the pattern of altered gene expression as affected tissues are supplied appropriate metabolic substrates.[20,21] Experiments involve a rat model with a 40% blood volume reduction over 10 minutes without resuscitation, followed by animal sacrifices at 1, 3, 6, 16, 24, 48, and 72 hours, where RNA is extracted from various organs (liver, lung, kidney, spleen, heart, and intestine). Changes of gene expression levels from each organ are obtained by comparing sham (control) and hemorrhaged rats using cDNA microarrays (GF300 cDNA filter arrays containing 5,140 rat genes; Research Genetics, Huntsville, AL).

Pioneering research undertaken by the Army and the Defense Advanced Research Projects Agency in this category also includes using gene expression arrays to study the wound-healing effect of light-emitting diodes (LED).[22] The goals of this study were to assess whether near-infrared LED light can indeed affect the wound-healing process and to identify the molecular mechanisms that might be involved. This research involves implanting polyvinyl acetal sponges in the dorsum of mice followed by daily exposures to LED treatment. Subsequently, the sponges, incision line, and the skin over the sponges are harvested to extract RNA and study the expression of various genes induced by the LED treatment. Preliminary studies revealed that certain tissue regeneration genes are induced by LED treatment when compared with untreated samples, suggesting that light therapy could enhance the regeneration process critical for wound healing.

Military Operational Medicine

DNA microarray research in military operational medicine is focused on two areas of unique military relevance: health effects of exposure to environmental toxic hazards and studies of exposure to environmental (heat and cold) stressors. The research on the health effects of organisms exposed to environmental toxicants is focused on the exposure to chemical compounds of military relevance, such as the ones found in explosives like 2,4,6-trinitrotoluene, 1,3-dinitrobenzene, and RDX (cyclonite). However, this research also benefits the public at large, as it is complementary to the efforts at the National Center for Toxicogenomics (NCT) of the National Institute of Environmental Health Sciences.[12,13,23] Similar to the NCT goals, the objective of the Army program is to identify and validate genetic biomarkers of exposure, effect, and susceptibility to various toxic hazards.

However, the Army is using a different strategy in toxicogenomics research. Taking advantage of ongoing public efforts to sequence species further down the phylogenetic scale, the approach is to use low-cost, nonmammalian "alternative species" such as worms (*Caenorhabditis elegans*), frogs (*Xenopus tropicalis*), and fish (*Danio rario* and *Oryzias latipes*) as model organisms to determine whether patterns of gene expression or protein biomarkers can reliably reflect the effects of toxic exposure and allow for interspecies comparisons and development of assays and bioreporter devices.[24–26] Initial efforts involve the exposure of *C. elegans* to low and high doses of chromium, 2,4,6-trinitrotoluene, and 1,3-dinitrobenzene followed by time course analysis using Affymetrix *C. elegans* GeneChip microarrays. This approach should translate into faster results at a reduced cost when compared with mammalian models (such as rats and mouse) used by the NCT and human studies. This work also builds on recent studies by Hamadeh et al.[12,13] suggesting that similar gene expression patterns can be associated with groups of chemical compounds according to their pharmacological and toxicological effects or modes of action. If these studies scale up as new chemicals are tested and indeed demonstrate that classes of chemicals share common gene response patterns, it will allow for the construction of an exposure database without the need to perform a chemical-by-chemical analysis, which would be prohibitively expensive and impractical.

To improve our understanding of soldier's tolerance and adaptation to exposure to heat and cold environmental stressors, the USAMRMC is performing in vitro gene expression studies with human cell lines separately exposed to heat and cold stress. A direct comparison of the genes affected by these two forms of thermal stress should reveal important similarities (stress-independent changes) as well as critical differences (stress-specific changes) in the transcripts involved, the direction in which they are changed, and the temporal pattern of expression from the time of exposure through the recovery period.[27,28] Ultimately, findings from these studies will be correlated with in vivo gene expression studies of soldiers who have suffered from exertional heat injury and hypothermia, and will result in an improved understanding and characterization of these cellular responses and the mechanisms by which they are regulated, leading to novel treatments and countermeasures to environmental stresses.

Medical Chemical and Biological Defense

Numerous studies are underway throughout the USAMRMC laboratories to catalog and analyze host gene expression responses to exposures to CWA and BWA. These studies involve a variety of in vitro experiments with different human and animal cell lines and in vivo experiments ranging from small mammalian models to nonhuman primates exposed to various bacteria, viruses, toxins, as well as blistering and nerve agents. Overall, the main goal of these studies is to gain insight into the cellular pathways and mechanisms of virulence and toxicity of the host response, leading to the discovery of agent-specific biomarkers for early diagnosis and the development of stage-appropriate prophylactic and therapeutic intervention strategies.

In medical biological defense, current host-response studies exploit the quick response of transcription factors to pathogen exposures and the fact that their time evolution reflects the course of illnesses after exposure. Traditional methods are based on the direct identification of the pathogen and, in general, are limited by a strong dependence on agent concentration, the time needed to reach detectable levels, the need for tissue sequestration of the pathogen, and the possibility of mutants (natural or deliberate) to escape detection. In contrast, studying the gene profile host response could provide the means for early diagnosis of exposure and the basis for designing stage-appropriate, therapeutic intervention strategies. Initial results of in vitro experiments using cDNA microarrays to study healthy human PBMCs exposed to approximately 12 different biological threat or pathogenic agents (e.g., *Bacillus anthracis*, *Yersinia pestis*, Venezuelan equine encephalitis, and botulinum toxin) suggest that unique gene expression signatures from different but related pathogens (such as anthrax strains) can be characterized, potentially leading to more reliable diagnostic assays.[29,30] These results are being confirmed using in vivo studies conducted with nonhuman primates, where correlations of gene profile responses at various stages throughout the study to the onset of the physiological aspects of illness can provide opportunities for understanding unknown gene functions and identifying stage-appropriate diagnosis and therapeutic strategies.[30]

The focus in medical chemical defense is on the applications of complementary genomics and proteomics technologies to the development of medical countermeasures against CWA. To date, there is no established medical treatment for CWA-induced injuries. Studies involving a variety of in vitro and in vivo human and animal models, including human cells, mouse, rat, pig, and guinea pig, are being performed to examine the biological responses of these models to lethal and nonlethal exposure to CWAs. Experiments have been performed with agents such as the blistering agent sulfur mustard[31–34] and organophosphonate nerve agents (e.g., VX, soman, and sarin).[35,36] The approach deployed in these studies combines the use of DNA microarrays monitoring the transcription-related changes with complementary two-dimensional gels and mass spectroscopy proteomics technologies. The objective is to isolate and identify specific genes and proteins that could help elucidate the responses and mechanisms of toxicity to CWA exposure and subsequent induced pathology, with the expectation that better understanding of these cellular and tissue pathways could lead to the development of prophylactic and therapeutic strategies. In particular, the hope is that these technologies will result in the discovery of specific biomarkers associated with CWA exposure pathology that can be used in drug target identification and to assess the effects of potential therapeutic drug treatments.[31,33]

One such application of gene expression technology in CWA research involves the study of pulmonary host responses to aerosolized toxins, such as ricin. As far as this particular agent is concerned, little is known of the role of gene regulatory circuits, and no effective preventive or therapeutic interventions are available. Two current experiments are underway, using mostly medium-density cDNA filter arrays (Clontech, Palo Alto, CA). In the first experiment, human pulmonary cells are exposed to various concentrations of ricin and are then treated with several drugs to test their ability to reduce ricin-induced cytotoxicity and identify genes involved in this process that could provide targets for potential drug screening.[37] In the second experiment, groups of mice are subjected to aerosol exposure to ricin and are then euthanized at specific time points (1, 4, 12, 24, 48, 96, and 192 hours) after exposure. Subsequently, their lungs are removed to extract RNA and to perform a histopathologic analysis.[38] The gene expression profiles of exposed animals are compared with those of control animals with the goal of identifying the differentially regulated genes responsible for tissue healing, inflammatory processes, apoptosis, and DNA repair. Specific aims include the determination of the molecular mechanisms of virulence/toxicity, the identification of host targets for developing countermeasures, and the development of diagnostic tools. Initial results indicate that transcripts could serve as early indicators of ricin-induced pulmonary toxicity, as specific genes respond many hours before any significant histological or physiological changes are observed.[38]

Technological Challenges Prevalent in Military Research

One may argue that the unique nature of military medical research does not necessarily lead to additional challenges of applying DNA microarrays that are not already experienced in civilian research. However, it is without doubt that the frequency and extent of such challenges in military medical research are more prevalent than in most civilian research and, in many cases, such challenges constitute an intrinsic part of the research. Furthermore, the military setting lends itself to performing certain experiments and collecting data not readily available in other settings. The key unique challenges and characteristics are discussed below.

Sparse Datasets

Experiment replication is the weapon of choice to account for the lack of precision (reproducibility) in DNA microarray experiments. In general, the number of replicate experiments is constrained by cost factors. Military medical research is often further constrained by the unavailability of data, leading to additional data analysis challenges. Examples of studies in which data tend to be sparse include exposure to high levels of environmental toxic hazards, BWAs, and CWAs, and extreme environmental conditions, where, unlike naturally occurring infectious diseases or neoplastic diseases, additional data cannot be collected using prospective studies. The challenge relates to the unfavorable relationship between the large number of di-

mensions of the problem (tens of thousands of genes) and the reduced number of samples. An additional and related challenge is the inability to design appropriate experiments, as often only sparse retrospective data are available. The question is whether any useful information whatsoever can be extracted from such limited microarray data.

Comparative Genomics Challenges

The limited availability or complete absence of in vivo human data adds additional challenges to the analysis of microarrays using comparative genomics. In such an instance, the effects observed using in vivo animal models and in vitro human data need to be projected to the effects in humans. Hence, a challenge is to extrapolate from these limited studies whole-body human effects while accounting for potentially not well-known or well-understood nonlinearities. A case in point is the research in BWAs and CWAs where, unlike naturally occurring infectious diseases, epidemic outbreaks are rare, requiring reliance on animal models that closely approximate humans, such as nonhuman primates.[30] This challenge is often aggravated, however, as the common mammalian animal models of choice (e.g., mouse and rat) for which genomic information is available do not respond like humans when exposed to certain CWAs such as nerve agents, requiring the use of animal models (e.g., guinea pigs and pigs) for which limited genomic information is available. Furthermore, for certain pathogenic agents, an optimal equivalent animal model may not exist, in which case, human in vitro studies are performed and extrapolated using sophisticated mathematical predictive models.

Lack of Appropriate Arrays

The reliance on animal models poses yet an additional challenge to the use of DNA microarrays. For some animal models, such as nonhuman primates, arrays are not available. The current solution is to use arrays constructed with human genes in experiments with animal mRNA samples. In this situation, the added challenge is to properly account for issues related to cross-species DNA binding in the analysis of the gene expressions.

Lack of Comparable Studies

In certain research areas, the military is the only institution conducting (one-of-a-kind) gene profile studies. Thus, there are no other institutions generating data that can be used to compare and validate the findings (gene expression, protein activity, and pathway interactions). This requires very solid internal validation and quality assurance procedures.

Access to Unique Datasets

Despite the added challenges discussed above, the military setting does provide certain unique advantageous characteristics allowing access to unique datasets and the performance of experiments that would otherwise be difficult to perform. One such example relates to the capability of performing longitudinal studies involving the same large number of volunteer soldiers over long periods of time. Another example relates to the capability of performing informed consent prospective human studies with soldiers that occasionally succumb to nonbattle injuries, such as exertional heat injury and hypothermia.

Summary

This review describes a representative cross-section of research projects exploiting DNA microarray technology that cut across the four medical research mission areas of the USAMRMC: military infectious diseases, combat casualty care, military operational medicine, and medical chemical and biological defense. It briefly discusses the scope, basic design, and aims of these projects, and identifies the major additional challenges of using microarray technology in military medical research. The USAMRMC and the research community at large should benefit from the findings summarized here, as this article provides increased awareness of the challenges and opportunities that lie within the realm of bioinformatics as it relates to analysis of gene expression array data and the particular Army needs in this field.

Although the challenges in the analysis of microarray data and the technology itself are daunting, they are, in general, not unique to the Army. They are symptomatic of a rapidly changing, emerging technology where evolutionary changes occur at a faster pace than the scientist's adaptation time. Although the USAMRMC should closely monitor progress in these areas, it should focus its resources in niche research areas and challenges that address unique military needs, such as (1) the development of a comprehensive database system of host gene exposure response to BWAs and CWAs (including combinations of dose amount and exposure elapsed time) that transforms dispersed biological threat resources into integrated and interconnected information resources. This system would allow for host-response comparisons across different exposure agents. (2) The pursuit of studies and analytical tools for identifying agent-specific biomarker signatures of particular BWAs, CWAs, and environmental hazard exposures that can uniquely discriminate, independent of dose and time, the source of the insult. This effort could allow for the determination of the existence and characterization of unique gene patterns associated with a given agent or class of agents. (3) The pursuit of studies and analytical tools for teasing out exogenous factors (such as operational stressors and ingested food) to allow for host gene response characterization of the original stimuli of interest (such as infected agent, chemical hazard, and environmental condition). This effort could potentially lead to the unique characterization of the insult of interest. (4) The pursuit of studies and the development of early diagnosis tools for differentiating naturally occurring infectious diseases from weaponized biological agents. This effort could potentially allow for early warning of a biological terrorist attack. (5) The development of analytical models that link gene expression responses to physiologic responses to allow for a deeper understanding of infection and disease pathology. This effort could lead to early diagnosis of disease and prediction of disease progression and treatment. (6) The pursuit of studies and the development of methods for the characterization of exposure to multiple agents. This effort could allow for the determination of the interaction and nonlinear effects of multiple agent exposure and the extent of the masking or overlapping effects on gene response.

DNA microarray technology is a powerful method that provides researchers with the opportunity to analyze the expression patterns of tens of thousands of genes in one experiment in a short time. However, when using microarrays, it is important to

keep in mind that this is a dynamic, young technology with a number of pitfalls[39] and, as such, the generated data need to be interpreted cautiously. Nevertheless, this and other emerging biological and biomedical research tools should not be ignored. As it was nicely put in a recent review addressing the revolution occurring in biotechnology,[40] "there are risks in signing up for the revolution, but there is also a greater risk in ignoring it—the risk of becoming uncompetitive."

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