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ABSTRACT

Title of Thesis: **"*In Vitro* Partial-Body Dose Assessment Using a Radiation Responsive Protein Biomarker"**

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Exposure of humans, accidental or occupational, to ionizing radiation poses significant health hazards. The objective of this study was to evaluate the response of a radiation responsive protein biomarker after a simulated partial-body ionizing radiation exposure. The radiation responsive protein biomarker studied was Growth Arrest DNA-Damage (GADD-45)α. For a 6-Gray gamma dose, the GADD-45α signal increased linearly as the percentage irradiated increased. These results support the use of molecular biomarkers to contribute in a multi-parameter diagnostic strategy for radiation exposure assessment.

Keywords: partial-body simulation, GADD-45α, microsphere-immunoassay, ionizing radiation, biological dosimetry, Luminex™

***In Vitro* Partial-Body Dose Assessment Using a Radiation Responsive Protein Biomarker**

By

Lt Jason M. Leidel

Thesis submitted to the Faculty of the Department of Preventive Medicine and Biometrics Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirement for the Degree of Master of Science in Public Health, 2005.

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Chapter 1: Background and Research Question

The exposure of humans to large doses, i.e. those greater than 0.1 Gray (Gy), of ionizing radiation, whether by accident or by occupation, poses significant health hazards. Identification, development, and validation of simple rapid detection and quantification methods are required to assess radiation dose to individuals in order to determine any applicable acute and long-term health risks. Human exposures to ionizing radiation are currently assessed by i) clinical signs and symptoms, ii) hematologic changes, and iii) chromosomal aberration analysis. Follow-on epidemiologic analysis of the above objective data will facilitate a health risk assessment. Clinical signs and symptoms are unreliable. Similarly, at lower doses (< 0.1 Gy) identifying stable, reproduceable hematologic changes are also challenging. Unfortunately, chromosomal-analytical methods are relatively slow, labor intensive, and time consuming making these techniques limited, particularly if field expediency is a necessary system requirement. However, recent technology allows the use of molecular biomarkers in various tissues and/or secretions (e.g., blood, hair follicles, saliva, buccal tissues, tears) as an innovative approach in applied radiation biodosimetry. Biomarkers are physiologic metabolites produced by environmental stressors or exposures (e.g. radiation) that can be readily identified using existing technology. The use of biomarkers may eliminate or minimize most of the limitations that are inherent with the use of cell culture-based chromosome aberration analyses. Recent work (Amundson *et al*., 2000) demonstrates the feasibility of using gene array technology in identifying sentinel radiation responsive genetic targets like the GADD-45α gene.

Growth Arrest DNA Damage (GADD-45)

There are three identified GADD-45 genes (α, β, and γ), which encode small (18 kilodalton, approximately $3x10^{-20}$g) evolutionary conserved proteins (proteins that have remained virtually unchanged during cellular evolution). These proteins are highly homologous to each other (i.e., 55-57% similar at the amino acid level) and are highly acidic (pH= 4.0-4.2). These proteins are primarily localized within the cell nucleus (Takekawa and Saito., 1998; Zhang *et al*., 1999). The GADD-45 genes are associated with the cellular response to repair DNA strand breaks and delay in cell-cycle progression (Yin *et al*., 2004). This study focused on the effect of radiation on GADD-45α protein response in human red blood cells.

Specific research objective

The purpose of this study was to determine whether or not a radiation responsive protein such as GADD-45α increases (up-regulation) after a simulated partial- and whole-body radiation exposure. Blood samples were obtained from a healthy donor and irradiated to a known 6-Gy dose. A mixture of irradiated and non-irradiated/ control blood (0-Gy from the same donor) was used to simulate partial-body dose fractions for 6-Gy exposures.

Motivation for this research

Diagnostic assessment of radiation exposure is necessary to support the triage of radiation casualties during peace and during times of conflict. A complete and accurate assessment is critical to the development of treatment strategies for individuals exposed to potentially life-threatening radiation induced injuries. To date, limited simulated

partial-body irradiation research has been accomplished. Therapeutic medical irradiations are generally partial-body. Additionally, no single biodosimetric assay is adequate to provide medical diagnoses for varied radiation-exposure scenarios as well as to provide surge-response capabilities due to mass radiological casualties (Prasanna *et al*., 2004). Accordingly, such a multiple parameter biodosimetry measurement tool with dual-use application (e.g., peace-time general medical care as well as war-time triage management) could significantly address military and civilian needs. The Armed Forces Radiobiology Research Institute's Biological Dosimetry Team (AFRRI-BDT) has pursued the identification and validation of protein markers for radiation dose assessment. These studies were designed to materially contribute to the development of a multi-parameter bioassay (signs/symptoms, hematologic and cytogenetic analyses, and molecular biomarkers) system designed to integrate biodosimetric diagnostic information in the medical management of radiation casualties (Sine *et al*., 2001; Salter *et al*., 2004). Specifically, this research aimed at determining if radiation-responsive biomarkers were up regulated in the presence of ionizing radiation during a partial-body simulation.

Research Question

Can radiation responsive up-regulation of molecular biomarkers such as the GADD-45α protein, be used to predict the whole-body dose delivered and/or to estimate partial-body dose received by the subject? If true, what is the nature of the relationship (e.g. linear, quadratic, logrithmic) between the level of candidate radiation responsive protein biomarker and the fraction of irradiated blood in a mixture?

Chapter 2: Literature Review

Biological dosimetry

Biological dosimetry relies on methods of quantifying radiation-induced changes at the molecular, cytogenetic, and cellular levels (Müller and Streller, 1991). The most advanced and reliable methods of biological dosimetry include; one, scoring of dicentrics (chromosomes having two centromeres) in peripheral blood lymphocytes (Bender *et al.*, 1988), two, the premature chromosome aberration assay for assessment in the clinically relevant high-dose exposure range (Prasanna *et al.*, 1997), and three, Fluorescent *In Situ* Hybridization (FISH) translocation assay in cases of prior-radiation exposure (Lucas *et al.*, 1992). Although significant improvements of protocols and instrumentation for chromosome aberration assays have been made, there is a need in radiation biodosimetry to develop an alternative diagnostic or triage screening radiation bioassay to address the high-sample throughput and rapid analysis requirements typically seen in mass radiation casualty scenarios. A situation where this would be advantageous or necessary would be if there was a large-scale radiation exposure. A multi-parameter bioassay strategy could be useful to meet these requirements. Donnadieu-Claraz's technique included demonstrating that some biochemical indicators showed significant elevations after radiation exposure (Donnadieu-Claraz *et al.*, 1999). For example, concentrations of amylase, alkaline and acid phosphatases, and iron were markedly higher after exposure. None of the studied parameters alone presents a reliable dose-effect relationship; however, there was evidence that the combination of lymphocyte and neutrophil counts and the determination of urea levels allowed some dose determination, independent of

time, if blood samples were taken within 7 days post-irradiation (Donnadieu-Claraz *et al.*, 1999).

The AFRRI-BDT initiated studies to develop a biodosimetry laboratory that combines analysis of both cytogenetic and molecular biomarkers with automated delivery platform configuration for rapid, high-throughput sample testing. Two candidate molecular bioassays to assess radio-responses have been identified and developed. Recent *in vitro* and *in vivo* studies have shown that several protein biomarkers are radio-sensitive (Miller *et al*., 2003; Blakely *et al*., 2003a; Blakely *et al*., 2003). Similarly, sentinel studies by Miller have also demonstrated radiation-responsive gene expression targets both *in vitro* and *in vivo* studies (Miller *et al*., 2002; Miller *et al*., 2003; Blakely *et al*., 2002a; Blakely *et al*., 2002b). Current measurements of protein biomarkers involve the use of a microsphere-based immunoassay, which provide high-throughput sample testing. This is true because these high-throughput machines such as the Luminex^{TM} can help analyze many more samples for multiple targets, at a fraction of the time.

New molecular biomarker based biodosimetry approaches

Molecular biomarkers are used as diagnostic endpoints in environmental health (Vainio 2001) and cancer (Preston 2002) risk assessments. For example, the blood level of prostate-specific antigen is a marker for prostate cancer. The human genome has some 50,000 to 100,000 genes that represent the template for many more proteins, generally with proteomic patterns specific to cell types and tissues. Biological monitoring of molecular biomarkers can provide radiation exposure assessment (Horneck, 1998; Becciolini *et al.*, 2001; Blakely *et al.*, 2001; Blakely *et al.*, 2002c, Blakely *et al.*, 2002b).

Although still in its infancy as a scientific discipline, the study of radiation biomarkers could include DNA mutations, gene expression, and protein endpoints. Cellular responses to ionizing radiation have been evaluated using gene-expression array technologies. A few highly over-expressing sentinel radiation-responsive targets were identified from an array of distinct gene-expression profile responses (Amundson *et al.*, 2000).

Biomarkers can be subdivided according to their applications (Brooks, 1999). For example, biomarkers of exposure and dose can be used to measure and reconstruct accidental or occupational radiation exposure when either limited data or no physical measurements are available. Similarly, biomarkers of risk or susceptibility could help to predict those individuals at greater risk for development of spontaneous disease.

Changes in gene and protein expression can be induced in peripheral blood lymphocytes by radiation exposure (Amundson *et al*., 2000). Current technology can detect radiation induced gene changes. The sensitivity of the gene expression bioassays appears to be adequate enough to detect gene changes at low radiation doses (0.2 Gy) (Amundson *et al.*, 1999). Using an *in vitro* human peripheral blood lymphocyte model system it has been recently demonstrated that both gene expression (Amundson *et al.*, 2000; Blakely *et al*., 2002a, 2003; Miller *et al.*, 2002; Grace *et al*., 2002, 2003); and protein (Blakely *et al*., 2003), changes following low-dose ($\leq$ 0.1 Gy) exposures remain elevated for longer periods of time (1-3 days). Further *in vivo* responses of several of these biomarker genes have been demonstrated in patients undergoing total-body irradiation (prior to bone marrow transplant) (Amundson *et al*., 2000). Use of a multi-target approach using genes based on DNA repair (i.e., GADD-45, DDB-2), apoptotic

(BAX, BCL-2), cell-cycle control (CDKN1A), and other radiation responsive pathways appear to show great promise in assessing radiation exposure. The gene expression changes shown in Figure 1 illustrate the dose response relationship for selected gene targets spanning delivered doses ranging from 0 to 1 Gy and ranging from 0 to 3 Gy using an *in vitro* human blood model system (Blakely *et al*., 2003). The diagnostically useful gene expression changes were measured using the quantitative real-time Polymerase Chain Reaction (PCR) methodology. The real time PCR instruments allow real time detection of PCR products as they accumulate during PCR cycles

Figure 1 Dose-responses of multiple gene expression targets

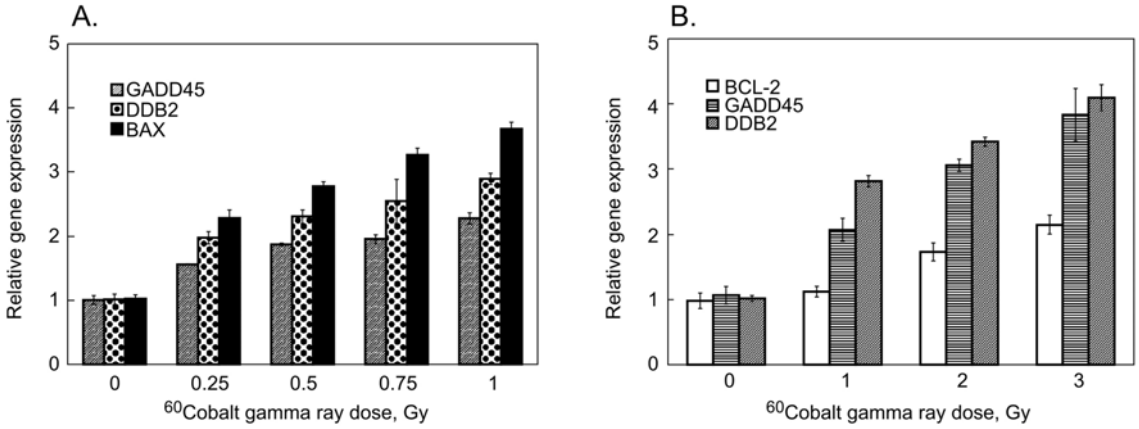


Figure 1. Dose-responses of multiple gene expression targets (GADD-45α, BAX, BCL-2, and DDB2) by multiplex real-time RT-PCR assay using an *in vitro* human blood model.

The data at 24 hours (Figure 1A) were obtained from a single RNA isolation of three replicates (R=3) per radiation dose from PCR assays performed on different dates. Three cDNA replicates (N=3) were PCR-amplified from each RNA isolate (R (3) x N (3) = 9) for each dose with individual gene targets. The x-axis illustrates the reported nominal doses of 0.0, 0.25, 0.50, 0.75, and 1.0 Gy at a dose rate of 0.1-Gy per min. The

y-axis shows relative gene expression, which is referenced to the 0-Gy (control) sample; error bars show standard errors of the mean. Dose response data at 48 hours (Figure 1B) for GADD-45α and DDB-2 targets were derived from three independent RNA isolation experiments to obtain mean-of-means data run in triplicate in three PCR assays, as described in Figure 1A. Initial BCL-2 data was derived from a single RNA isolation and one PCR assay (R x N=9 per dose). The x-axis depicts the reported nominal doses of 0.0, 1.0, 2.0, and 3.0 Gy at a dose rate of 0.1-Gy per min. (Blakely *et al.*, 2003).

The use of protein biomarkers as potential diagnostic tools is an emerging area of science that is being intensively investigated in carcinogenesis, toxicology, radiation exposure assessment and other diseases. The association of biologic markers with cancer has been recognized for many decades (e.g., Gutman *et al.*, 1936). Prostate-Specific Antigen (PSA) is the most widely used tumor marker and serves as a benchmark for the study of other tumor markers (Israeli *et al.*, 1997). More interestingly, a number of radiation-responsive protein biomarkers have been documented from the literature (Table 1). As stated earlier, these markers can be used to measure and reconstruct exposures and doses but likely do not indicate susceptibility of a higher risk of developing a spontaneous disease. Background studies and evidence supporting the feasibility of the proposed research work come from various experiments done in collaboration of research groups at AFRRI. These pilot studies were the proof-of-concept for the proposed protein biomarker project.

Table 1 Selected list of radiation-responsive protein biomarkers and their respective tissue or cell location

Protein	Localization (Tissue/Cell)	References
Alkaline phosphatase	Blood	Donnadieu- Claraz *et al.*, 1999
Amylase	Parotid gland	Dubray *et al.*, 1992
Cytokines (IL-6, TNF-α)	Skin and blood cells	Beetz *et al.*, 1997
GADD-45 and proto-oncogenes	Blood	Papathanasiou *et al.*, 1991
Substance P	Parotid gland	Aalto *et al.*, 1995

Figure 2 Proto-oncogene and DNA repair protein expression

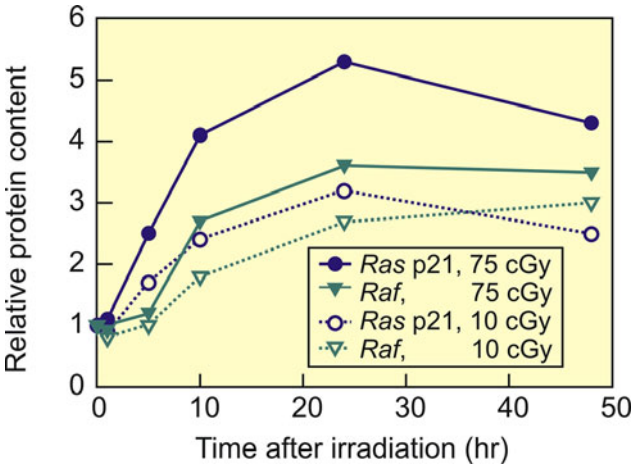


Figure 2: Time course of ras p21 and raf protein content in human *in vitro* blood lymphocyte model.

This blood lymphocyte model (Figure 2) involved *in vitro* exposure to 0.1 and 0.75 Gy 250 kVp x-rays (1.0 Gy/min). Samples were derived from the blood lymphocyte cell pellets. Protein content was determined by spectro-photometric analysis. Specific protein biomarkers were detected at equivalent total protein levels using a conventional ELISA method. Symbols represent the means (n = 5, SE were < 20% of the means) for ras p21 (circles) and raf (triangle) after exposure to 0.1 (open circles) and 0.75 (solid

circles) Gy. Components of these results are derived from previous published studies (Miller *et al*., 2002; Blakely *et al*., 2003).

Figure 3 Radiation-responsive changes in the expression of ras-p21 and p21 Waf1Cip1 in peripheral blood serum

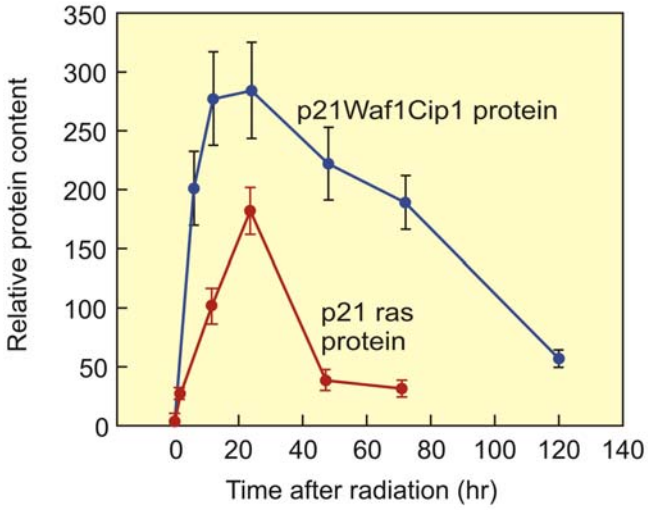


Figure 3: Radiation-responsive changes in the expression of ras-p21 and p21 Waf1Cip1 in peripheral blood serum.

For Figure 3, the radiation responsive changes are from 0.25-Gy irradiated rodents. Each symbol represents the mean from 5 to 12 animals. The results shown in this figure demonstrates that p21Waf1Cip1's relative protein content was more up-regulated compared to the p21ras protein. Components of these results are derived from previous published studies (Miller *et al*., 2002; Blakely *et al*., 2003).

Figure 4 Radiation dose-dependent increase of GADD-45α in serum samples

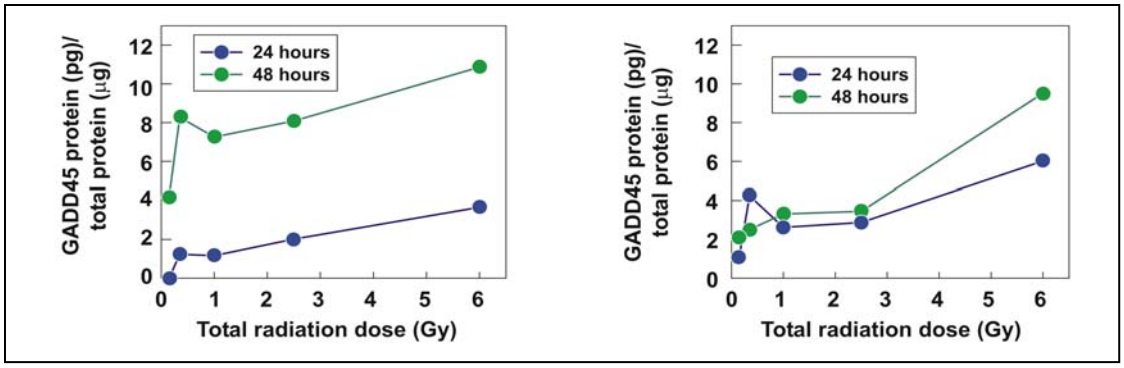


Figure 4 Radiation dose-dependent increase of GADD-45α in serum samples **(**Left Panel) and cell pellets (Right Panel) using a human *in vitro* blood model system. Encoded protein was detected using the microsphere immunoassay (Luminex^{TM}) methodology (Muderhwa *et al.*, in preparation).

AFRRI-BDT initially intended to focus on evaluating the radio-response of GADD-45α and amylase. Ultimately, they envisioned the use of multiple biomarkers (Table 2) to accurately determine exposure dose. Samples will be archived for later analysis of additional candidate radiation responsive biomarkers.

Table 2 Selected list of candidate radiation responsive targets

Target class	Proposed blood protein targets
DNA damage & repair	GADD-45
Cell cycle and cell proliferation	CDKN1A (p21CIFWaf)
Apoptosis/Anti-apoptosis	To be determined
Proto-oncogene	RAS (p21ras)
Tissue injury markers	Amylase (salivary gland, acinar cells), tissue polypeptide antigen or TPA (salivary gland, ductal cells); intestinal alkaline phosphatase

Figures 1 through Figure 4 demonstrate that gene and protein content may increase for certain radiation responsive biomarkers. However, the previous work has only focused on whole-body exposures. This researched focused on a partial-body simulation. Figures 2 and 3 demonstrate that the largest relative protein content was found approximately 24-hours after irradiation. This piece of information was used when deciding how long to allow for repair after irradiation. Figures 2, 3, and 4 illustrate that there is a decrease at the 48-hour time point relative to the 24-hour time point for most of the biomarkers.

How radiation effects protein biomarkers

Ionizing radiation induces several cellular responses to include DNA damage, changes to protein biomarkers, and tissue inflammation/injury. Alterations of cellular components following radiation exposure suggested a potential use of proteins as biomarkers of radiation exposure. It is well established that cells are subject to arrest at G1 (Gap 1) and G2 (Gap 2) checkpoints in response to DNA damage, presumably to allow time for DNA repair prior to entry into the S (synthesis) and M (mitosis) phases, respectively (Murray 1992). The p53 tumor suppressor is required for one such G1 checkpoint and functions to up-regulate expression of GADD-45α and p21 (Kuerbitz *et al.,* 1992 and Kastan *et al.,* 1992). The p21 gene functions to inhibit the kinase activity of multiple Cyclin dependent kinase (Cdk) complexes, which may account for its suppression of cell growth (Michieli *et al.*, 1994, and El-Deiry *et al.*, 1994). The GADD-45 genes bind to both Cdks and proliferating cell nuclear antigens (PCNA), which is a protein involved in DNA replication and repair (Smith *et al.,* 1994). Therefore, it has been suggested that GADD-45 may serve as a link between p53-dependent cell-cycle checkpoints and DNA

repair (Marx 1994). This means that up-regulation of GADD-45 should occur when exposed to ionizing radiation because ionizing radiation causes DNA damage. Therefore, more DNA damage implies greater potential for DNA repair. This project expected that an increase in the percentage of irradiated blood would linearly increase the relative GADD-45α protein signal.

Chapter 3: Methods

Experimental design and method

Blood samples obtained from a healthy-adult human subject were used after obtaining informed consent. The blood was irradiated at 0.1-Gy/minute for 60 minutes giving a total dose of 6-Gy from ^{60}Co gamma rays (two mono-energetic rays of 1.17 MeV and 1.33 MeV). The same donor was used for each of the three replicates to control for inter-individual variability. The informed consent process and form were approved by the Institutional Review Board of the Uniformed Services University of the Health Sciences.

Experimental description

Blood was aseptically drawn from a healthy male 24-year old donor via venipuncture using 9 collection tubes (~38 ml) with lithium heparin to prevent coagulation. After inverting several times (to prevent clotting), the blood was placed into 15-ml conical tubes (only half full) and the tubes were labeled and separated into two groups—experimental (irradiated) and control groups. For consistency, both the control and experimental tubes were treated and handled the same with the obvious exception that the experimental group was given a dose of 6-Gy at 0.1-Gy per minute.

During this one-hour irradiation, 1.5-ml and 15-ml test tubes were being labeled. The labels for the 1.5-ml test tubes included group (percent irradiated), replicate number (1, 2, or 3), and serum versus cell pellet. The labels for the (six) 15-ml conical tubes included just the group letter. It should be noted that the AFRRI-BDT uses different terminology than the norm. Serum equates to the blood plasma and white blood cells and

the cell pellet is the red blood cells. An example of how these tubes were labeled would be "A, 1, P." This means group A (control or 0% irradiated), replicate number 1, and P is the cell pellet. The 1.5-ml test tubes were used in the next day for the partial-body simulation.

After approximately one-hour of irradiation, the conical tubes were collected and taken back to AFRRI room 1313 for the partial-body simulation. An equal volume (5-ml) of pre-warmed (37^{o}C) Roswell Park Memorial Institute media (RPMI) plus 10% fetal bovine serum media was added to each 15-ml conical tube. This media allows for an environment comparable to the human body. The blood was then vortexed for approximately 10 seconds to ensure thorough mixing.

The next step was the partial-body simulation. The goal was to add approximately 12-ml to each of the six newly labeled conical tubes. See Table 3 for the exact mixture for each group. The partial-body simulation was incubated with the cap loosely fastened, angled at 45^{o} and allowed to repair for 24 hours in a 37^{o}C incubator with 5% CO_2. After 24 hours, the conical tubes were removed from the incubator and vortexed again. The conical tubes were then centrifuged for 10 minutes at 2400 rpm (610 x gravity) at 24^{o}C. The supernatant (serum/ plasma ~ 60% of the total volume) and blood cell pellet were transferred to the appropriate test tubes with any excess kept for future analysis. When finished transferring the blood to the appropriate tubes all test tubes were placed at -80 ^{o}C.

Total protein amount in detergent (MEM-Per Reagent A, Pierce Chemical Co.) treated blood pellet samples were measured using the copper reduction/bicinchoninic acid (BSA) reaction kit (Pierce Chemical Co.) as described earlier (Smith *et al*., 1985)**.**

GADD-45α measurements in the simulated partial-body exposure were analyzed via Luminex™ technology. The major difference between the Luminex™ and ELISA processes is the location of the bound antibody. When using the Luminex™ methodology the primary antibody is bound to Luminex™ beads, whereas for the ELISA the antibody is bound to the 96-well plate. For an over-view of how the Luminex™ capture antibody works please see Figure 5. For a detailed description of the differences between Luminex™ vs. ELISA see Appendix 3. First, the test tubes were removed from the freezer and allow to thaw. Then, a 96-well filter-bottom micro-plate, which was pre-wet with 200 micro-liter of Assay Buffer Solution, was obtained. Next, Luminex™ bead set #32, which were conjugated with GADD-45α capture mouse monoclonal antibody (Santa Cruz catalogue number 4T-27:sc-796), was added to each well (40 µl~5000 beads). Then, 125 µg of total protein was added to each well of a 96-well plate. Each dilution was normalized to 125 µg of total protein. A rabbit polyclonal biotinylated detection antibody coupled with biotin (Santa Cruz catalogue number H-165: sc-797) was placed into the reaction to bind to the analyte. The next step was the addition of 40 µl Streptavidin: R-Phycoerythin. Streptavidin binds to biotin with high affinity. Finally, Streptavidin-Phycoerythrin (PE) was used for detecting biotinylated antibodies. The sample was then analyzed in the Luminex™ flow cytometer (Luminex™ model XYP, Austin, TX) equipped to analyze the labeled conjugated beads. The excitation of PE by 480 nm laser light induced a light emission maximum of 578 nm. This light emission was what gave the optical density or relative fluorescence, which was used to generate data from Luminex™ XYP. For additional information on how flow cytometry works, see reference by Wallace (2005).

There are three isoforms of GADD-45: α, β, and γ. An isoform is any of two or more functionally similar proteins that have a similar but not identical amino acid sequence and are either encoded by different genes or by RNA transcripts from the same gene which have had different exons removed (Merriam-Webster 2005). An exon is a sequence of DNA that codes information for protein synthesis that is transcribed to messenger RNA (Merriam-Webster 2005).

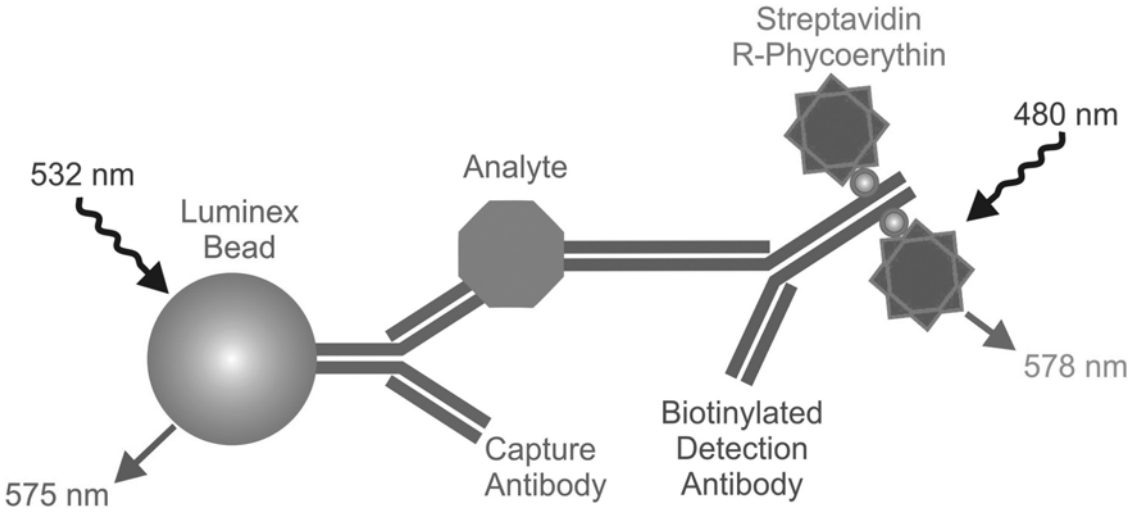
Data analysis

The optical density was analyzed using the software package Luminex™ Data Collector Version 1.7 provided by Luminex™. This software outputs a relative GADD-45α protein signal, which was used for data analysis. Three independent experiments were performed with 36 data points per percentage irradiated per replicate. The data was graphed and was fit to a linear model using least squares regression analysis via the Statistical Package for the Social Sciences (SPSS, Chicago, Il).

Table 3 A chart representation of the experimental design

Partial-Body ID (% and group letter)	Percentage		Incubation time after exposure (hours)	No. of replicates per sample	No. of experiments
	Irradiated	Control			
0/ A	0	100	24	3	3
20/ B	20	80	24	3	3
40/ C	40	60	24	3	3
60/ D	60	40	24	3	3
80/ E	80	20	24	3	3
100/ F	100	0	24	3	3

Figure 5 LuminexTM sandwich capture antibody schematic



from Vignali 2000

Figure 5 A pictorial representation of the reagents used in this assay. Wavy lines represent the excitation light beam, straight lines the emission beams. The 575 nm emission beam was evaluated to quantify the GADD-45α (analyte).

Chapter 4: Results

In vitro human whole blood was exposed to 6-Gy (0.1 Gy/min) of bilateral Cobalt-60 gamma radiation and mixed with non-irradiated blood from the same donor. The blood was mixed in such a way to simulate partial-body exposures. The samples were allowed to repair for 24 hours before being analyzed via the Luminex-100^{TM} system (model XYP). Three independent experiments were performed. The symbols represent the means of each replicate experiment. Standard errors of the means are less than the symbol size (Figure 5). The data was fit to a linear model ($y = mx + b$) using a least squares regression analysis.

The over-all fit of this model was very good. The R^2 value was consistently around 0.998. Analysis of variance was used to examine the relationship between relative GADD-45α protein and percentage of blood irradiated. As illustrated in Figure 6 and in Table 4 a significant relationship between the relative GADD-45α protein content and the percent irradiated was supported using this bi-variate analysis.

Figure 6 A graphical representation of the results from the GADD-45α *in vitro* partial-body radiation simulation

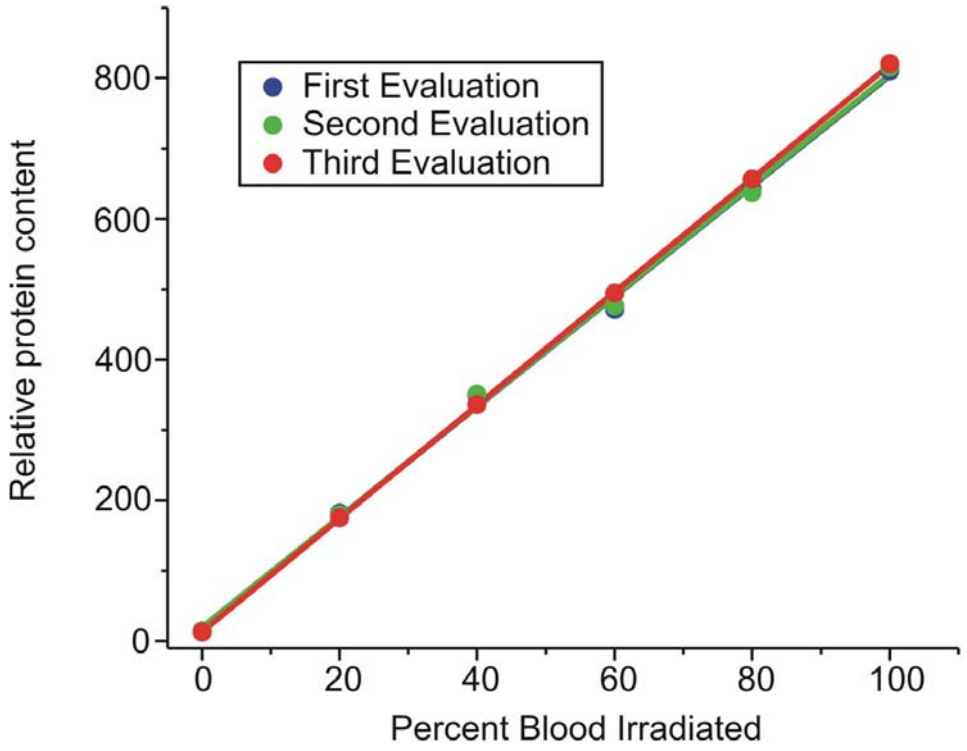


Figure 6 GADD-45α protein response as a function of percent human blood irradiated (6-Gy) in simulated partial-body exposures.

Table 4 Statistics supporting GADD-45α up-regulation in partial-body simulation

	R^2	F	p	Equation of line Y = mx+b (including +/- SE)	Statistically Significant?
Replicate 1	0.999	315481.99	< 0.001	RPC = (8.1 +/- 0.14)(PBI) +(0.51 +/- 0.87)	YES
Replicate 2	0.997	74794.935	< 0.001	RPC= (7.8+/- 0.29)(PBI) +(7.2+/- 1.7)	YES
Replicate 3	.998	86324.552	< 0.001	RPC=(7.9+/- 0.27)(PBI) +(7.1 +/- 1.6)	YES

RPC= Relative Protein Content PBI= Percent Body Irradiated

In analyzing the data, the background level from the control wells was first removed or subtracted from the total Luminex-100^{TM} signal. Figure 6 shows the linear relationship between each of the data points. The experiment was performed three times,

with samples drawn from the same donor thus allowing for a numerous amounts of data per point. All results were placed on one graph for inter-comparison of the data. For additional information please see Appendix 2.

Chapter 5: Discussion and Conclusion

The GADD-45α gene was studied because it was the first commercially available antibody for this protein. In addition, Muderhwa (2003) performed sentinel studies with GADD-45α that indicated a preliminary radiation response existed between dose and up-regulation of this protein.

This project focused on GADD-45α, and it was determined that GADD-45α up-regulation occurred following 6-Gy irradiation and also showed a linear dependency based on the percentage of irradiated blood in the simulated partial-body exposure. This assertion was made because the R^2 value was near 0.998 for all three replicates. A value of 1.0 is a perfect straight line. This indicated that the linear regression model was the best fit for the data and that by inspection of Figure 6 the relative protein content increased with the increased percentage of irradiated blood. The equations for each line are listed in Table 4. These equations can be used to predict other percents of blood irradiated. This experiment focused on a gamma ray dose above the known lethal dose (4.5-Gy) at 50% (LD_{50}) for humans.

This up-regulation validates the hypothesis. As a larger percent of irradiated blood was presented in the mixture, an increased GADD-45α signal also occurred. By inspection of Figure 6, one can see that lowest point is the 0% (non-irradiated) and the highest point is the 100% irradiated blood. Thus, when simulating partial-body irradiation, one should expect an increased signal from a radiation responsive protein as more of damage cells were introduced to the mixture.

The bystander effect was a possible confounder if the GADD-45α up-regulation results were not linear. The bystander effect is primarily a phenomenon in which, for example, cells that are damaged by radiation are thought to send out signals to

neighboring cells. These signals can cause cellular damage or dysfunction beyond the scope of the original radiation insult. Most studies of the bystander effect are limited to fairly high doses of alpha radiation, given over a very short period (Hall and Mitchell 2003). Because the cells were irradiated with gamma rays, and mixed with control blood, the bystander effect was still considered a viable option. This mixing could have allowed for the cells damaged by radiation to communication with non-irradiated cells and cause secondary damage. The results of the study demonstrated a linear relationship between GADD-45α protein up-regulation and fraction of irradiated blood, hence, there is no need to evoke a significant role of the bystander effect in this study.

Conclusion

At a Co-60 gamma ray 6-Gy dose the GADD-45α fluorescence signal increased with an increased GADD-45α percentage of irradiated blood for the *in vitro* partial-body simulation study. This technique shows repeatability for this one donor and based on the equation of the line(s) can predict GADD-45α signal for any percentage irradiated for that replicate. However, this conclusion is limited to the one donor at 6-Gy. Based on these results, it is envisioned that at other doses for a partial-body simulation, a linear increase would also occur for this donor.

The following recommendations are made to facilitate further research:

(1) Use more than one donor and evaluate for potential inter-individual variability,

(2) Design and evaluate the dosimetry of the murine *in vivo* partial-body irradiation set-up,

(3) Demonstrate the utility of radiation responsive protein bioassay to assess partial-body exposures in a murine *in vivo* validation,

These projects would offer a quantitative method to determine an up-regulation of radiation responsive proteins. A proteomic (protein) diagnostic method compliments alternative methods of exposure assessment (i.e. time to and severity of vomiting, and diarrhea). This quantitative method may bridge the gap between the latency period low-dose radiation exposure, and near real-time (24 hour) diagnostic assessments. If so, it would reinforce the notion of developing a high-throughput multi-parametric biodosimetry assay system compatible with military field laboratories, homeland security applications and radiation therapy centers to quickly assess radiation exposure based on blood protein biomarkers capability.

Assessment of soldier's radiation exposure will support commander's tactical operations and medical decisions. This strategy addresses the need for a "*Field Radiological Biodosimetry*" system and promotes effective command decisions and force structure planning to ensure mission success. In addition, assessment of a population's exposure in other radiation threats, such as nuclear accidents and terrorism mass casualty scenarios addresses the need for a "*Clinical Radiological Biodosimetry*" system to provide physicians with the ability to triage radiation victims, make appropriate treatment decisions, and reduce uncertainties associated with the variability of individual response to radiation exposure.

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Appendix 1

Future Considerations

Specific Aims.

It is recommended that this research be continued initially involving three specific aims.

i) Use more than one donor and evaluate for potential inter-individual variability (Specific Aim 1). It will also be interesting to see how this technique responds to different radiation doses. A future study could be to take blood from radiation therapy patients involving partial-body irradiation procedures both before and after irradiation. This would allow for each person to serve as their own control. Using the method outlined in this thesis on various donors with different demographics is suggested.

ii) It is also recommended that for Specific Aim 2 one would design and evaluate the dosimetry of the murine *in vivo* partial-body irradiation set-up. Thermo-luminescent dosimeter (TLD) chips or Optically Stimulated Luminescent (OSL) should be placed near the mice in the plexiglass tubes in order to measure the dose profile to the mice. GAFCHROMIC film is recommended with a densitometer to measure the uniformity of the radiation. This would allow identifying and characterizing any "spill-over" radiation into areas that were thought to be shielded during the partial-body irradiation.

iii) Next, demonstrate the utility of radiation responsive protein bioassay to assess partial-body exposures in a murine *in vivo* validation (Specific Aim 3). The mice should be divided into three groups for irradiation (head only, head and chest, and whole body) to a

dose of 6-Gy. Blood should be collected from the irradiated mice and compared to the *in vitro* human blood simulation.

Suggested methodology

Specific Aim 2 Design and evaluate the dosimetry of the murine *in vivo* partial-body irradiation set-up

Experimental design

Four mice holders with mice or mice phantoms will be placed in a row between lead bricks such that appropriate percentage of the mouse will be exposed to the gamma field (see Figure 9). The dose rate will be 0.6-Gy/min for ten minutes for a total dose of 6-Gy. For safety reasons the lead bricks will be placed on the main experimental table without any vertical spacing.

Dosimetry measurements

(TLDs) chips or (OSLs) will be placed near the mice in the Plexiglas tubes in order to measure the dose to the mice. TLDs or OSLs will be placed in the proper sections of the phantoms to measure field uniformity. GAFCHROMIC film will be used with a densitometer to measure the uniformity of the radiation as well as the distribution of the dose. As a further verification, a densitometer will be used to evaluate GAPHCHROMIC film dose distribution.

Suggested dosimetry set-up

Table 5 A chart representation of the experiment design for specific aim 2

Head

Position	TLD/ OSL	film	Number of replicates
Head	Yes	Yes	3
Shoulder	Yes	Yes	3
Hip	Yes	Yes	3
Tail	Yes	Yes	3

Head and Torso

Position	TLD/ OSL	Film	Number of replicates
Head	Yes	Yes	3
Shoulder	Yes	Yes	3
Hip	Yes	Yes	3
Tail	Yes	Yes	3

Whole Body

Position	TLD/ OSL	Film	Number of replicates
Head	Yes	Yes	3
Shoulder	Yes	Yes	3
Hip	Yes	Yes	3
Tail	Yes	Yes	3

Figure 7 Shielding design for the *in vivo* validation

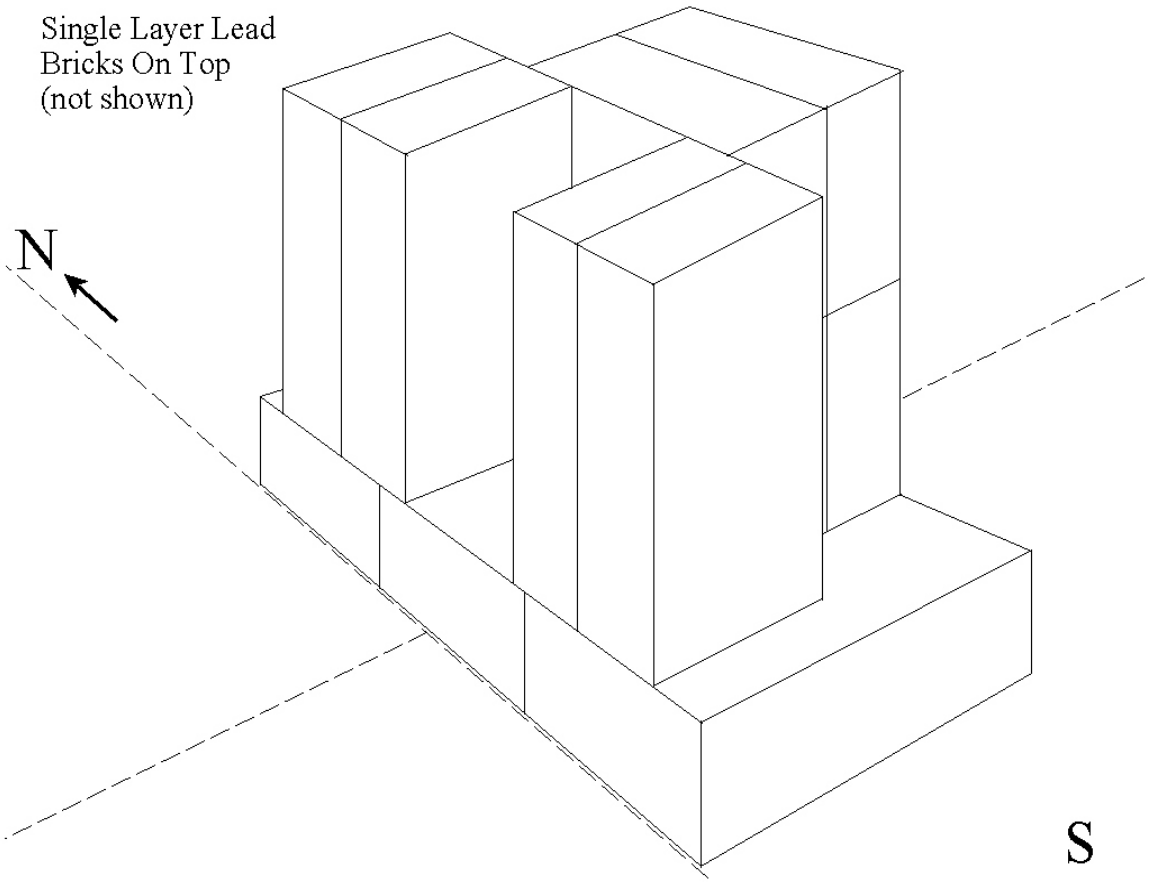


Figure 8 A schematic of 4 mice in holders

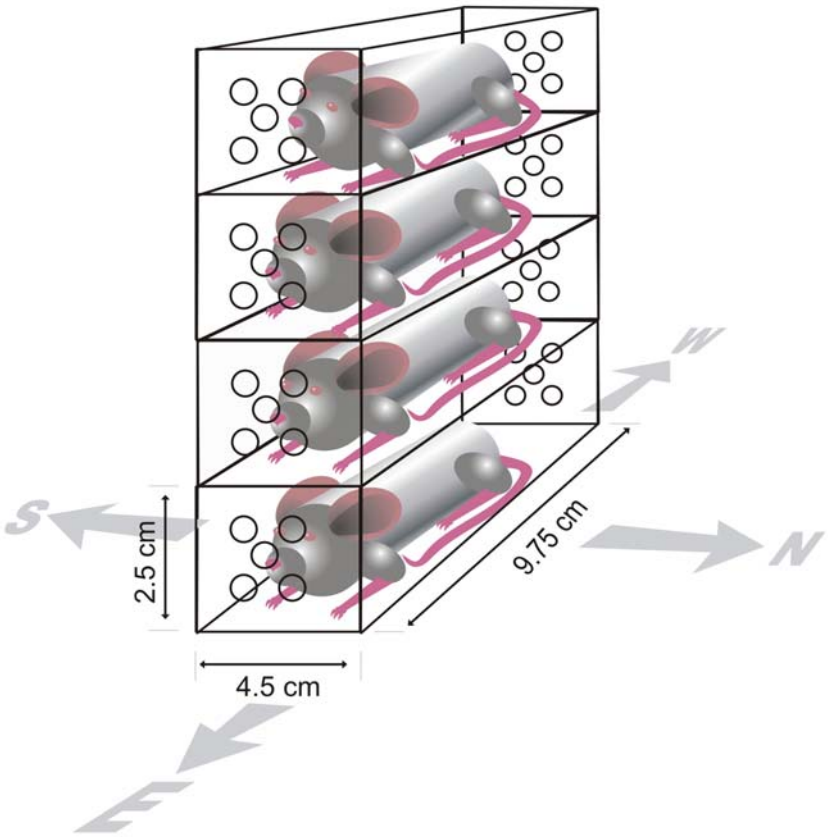


Figure 9 A schematic of mice phantoms in holders

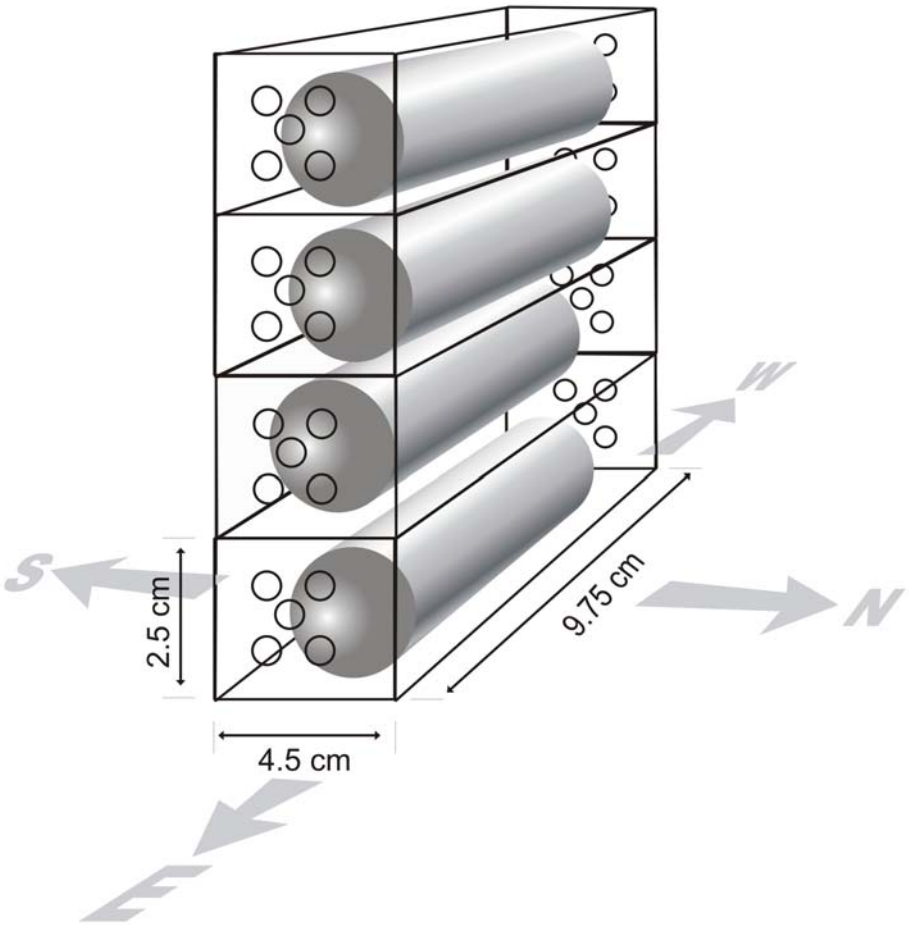


Figure 10 A schematic of 1/3 partial-body mice and phantom irradiation

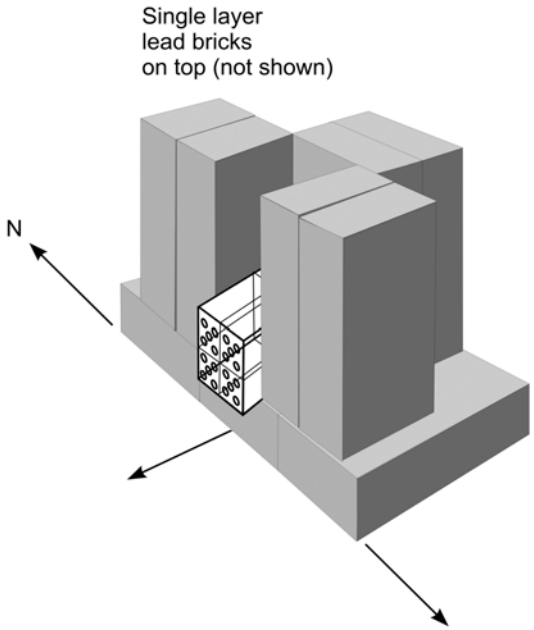


Figure 11 A schematic of 2/3 partial-body mice and phantom irradiation

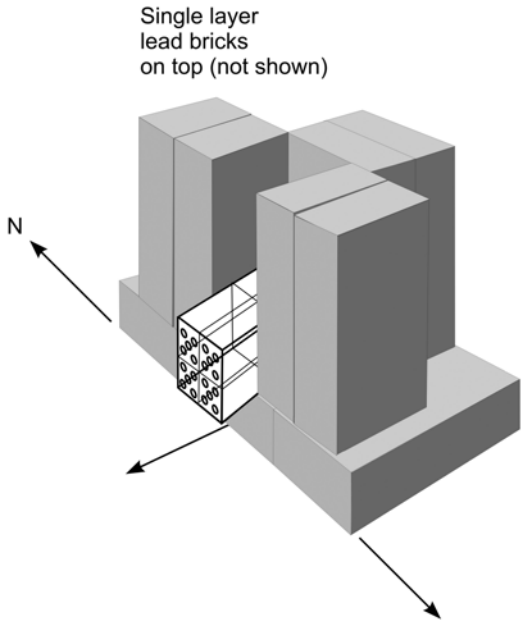
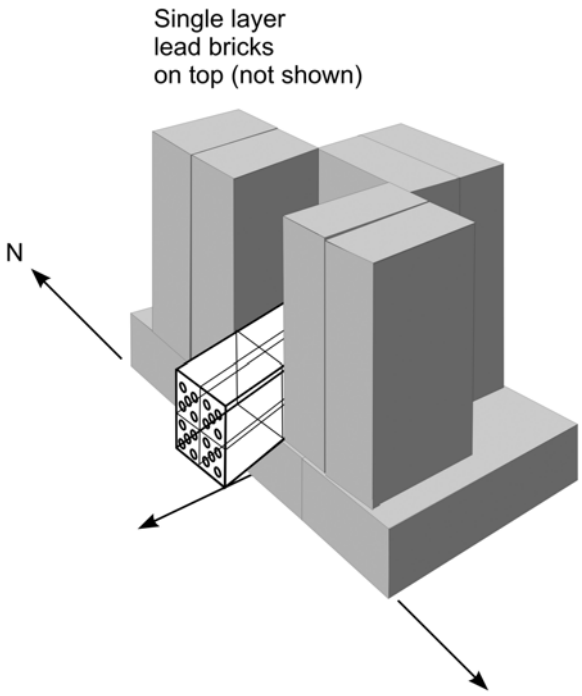


Figure 12 A schematic of whole-body mice and phantom irradiation



Specific Aim 3 Demonstrate the utility of radiation responsive protein bioassay to assess partial-body exposures in a murine *in vivo* validation.

Experimental design

The proposed experimental design involves male mice of the Balb/c strain and comparable phantoms irradiated at a known dose (6 Gy @ 0.6-Gy/min for 10 minutes**)** to a known portion of their bodies (one-third, two-thirds, whole body). The samples will be in triplicate (three replicates) for each fraction as well as enough controls to have statistical significance. Mouse blood will be collected after 24 hours via terminal cardiac puncture from the irradiated mice after using VSD recommended pain relief protocol. The mice will then be euthanized via a carbon dioxide induction chamber. The radiation responsive protein levels will then be evaluated in mice blood samples.

Balb/c strain will be used in this study. This mouse strain was chosen based on preliminary encoded protein data (Miller *et al*., in preparation). The mouse model was chosen as the best species for this *in vivo* validation study for the following reasons: (i) Qualitative similarities exist between human and mouse proliferative tissue including bone marrow (Thompson *et al.,* 1962). (ii) Molecular responses of mouse and HPBL (human peripheral blood lymphocytes) to gamma radiation are expected to be similar. (iii) The ease of use of a mouse model system ensures reliable data collection. (iv) Median lethal dose ($LD_{50/30}$) for radiation-induced death for Balb/c mice is known to be 5 Gy (Dr. Alexandra C. Miller personal communication citing her thesis). This information is necessary for extrapolating data to the human population. The model provides experimental and statistical validity.

Biosamples

Two time points were selected to compare results with *in vitro* results. Blood sampling at 24 and 48 hrs, in separate groups of animals, were selected for this study based on radiation accident operational considerations. For example, generic guidelines recommend that blood for cytogenetic analysis be collected 24 hr after a suspect radiation exposure. We have also established GADD-45α dose responses using a human *in vitro* blood model at 24- and 48-hr. Prior to conducting the experiment on Groups A through F, a small pilot study will be conducted on groups "Pilot 1" and "Pilot 2" (See Table 6). These animals will undergo the same exposure and sampling procedures as Group A and D, however blood collected during the pilot study will be used to verify and perfect anesthesia, lab technique, and sample size requirements for the Balb/c strain. The use of 32 mice (Table 5, Pilot 1 and 2) is justified for the purposes of empirically confirming the projected sample size requirement for the planned study. During the time leading up to the experiment, other sources of Balb/c blood will be sought out through the AFRRI Tissue Sharing program so that animals described in the table may not be needed for this pilot study. Group E and F permit evaluating the potential confounding effects of anesthesia (Jacobsen *et al.*, 2004) on the proposed research study.

Table 6 A chart representation of the experiment design for specific aim 3

Position	Anesthesia for restraint during radiation	Portion of body irradiated	Estimate of percentage of the body irradiated (%)	Blood Draw post-exposure (hours)	No. of animals per sampling time	No. of experi-ments	Total number of animals
Pilot 1	Yes	None/ Control	0	24, 48	8	1	16
Pilot 2	Yes	Whole	100	24, 48	8	1	16
A	Yes	None/ Control	0	24; 48	8	3	48
B	Yes	Head Only	33	24, 48	8	3	48
C	Yes	Head and Torso	67	24, 48	8	3	48
D	Yes	Whole Body	100	24, 48	8	3	48
E	No	Whole Body	100	24, 48	8	3	48
F	No	None/ Control	0	24, 48	8	3	48
						Total number of animals	320
						VSD Quality Assurance	7
						Total Number of Mice	327

Appendix 2

SPSS Analysis

Table 7 Analysis of GADD-45α in vitro partial-body simulation for replicate #1

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	1.000(a)	.999	.999	7.203

a Predictors: (Constant), adj_resp

ANOVA(b)

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	16368409	1	16368409.289	315481.99	.000
	Residual	11103.137	214	51.884		
	Total	16379512	215			

a Predictors: (Constant), adj_resp
b Dependent Variable: response

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
			Std. Error	Beta			Lower Bound	Upper Bound
1	intercept	.511	.869		.588	.557	-1.201	2.224
	slope	8.059	.014	1.000	561.678	.000	8.031	8.088

a Dependent Variable: response

Table 8 Analysis of GADD-45α in vitro partial-body simulation for replicate #2

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.999	.997	.997	14.3851

a Predictors: (Constant), pct

ANOVA(b)

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	15477338.322	1	15477338.322	74794.935	.000(a)
	Residual	44283.084	214	206.930		
	Total	15521621.406	215			

a Predictors: (Constant), pct
b Dependent Variable: adj_resp

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
			Std. Error	Beta			Lower Bound	Upper Bound
1	intercept	7.214	1.735		4.158	.000	3.794	10.635
	slope	7.837	.029	.999	273.487	.000	7.780	7.893

a Dependent Variable: adj_resp

Table 9 Analysis of GADD-45α in vitro partial-body simulation for replicate #3

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.999(a)	.998	.998	13.4337

a Predictors: (Constant), pct

ANOVA(b)

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	15578600.251	1	15578600.251	86324.552	.000(a)
	Residual	38619.609	214	180.465		
	Total	15617219.860	215			

a Predictors: (Constant), pct
b Dependent Variable: adj_resp

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients			95% Confidence Interval for B	
			Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
1	intercept	7.022	1.620		4.334	.000	3.828	10.217
	slope	7.863	.027	.999	293.810	.000	7.810	7.915

a Dependent Variable: adj_resp

Appendix 3

ELISA vs LuminexTM

Muderhwa *et al*. (2003) proposed to develop a radiation-responsive blood-protein biomarker analytical system based on conventional capture sandwich immunoassay. The conventional sandwich immunoassay of choice is the Enzyme-Linked Immunosorbent Assay (ELISA). This assay is used to determine the antigen concentration in unknown samples. This ELISA is fast and accurate, and if a purified antigen standard is available, the assay can determine the absolute amount of antigen in an unknown sample. The sandwich ELISA requires two antibodies that bind to epitopes (the ends of the antibody) that do not overlap on the antigen. This can be accomplished with either two monoclonal antibodies that recognize discrete sites or one batch of affinity-purified polyclonal antibodies. The ELISA is a fundamental tool of clinical immunology. The first step is to coat each well (of a 96 well plate) with the antigen of choice. If the same contains these antigens they will bind to the wells (if not, then they will not bind). Another antibody is coupled to an enzyme. This is the second antibody, and it binds to the antibodies that are being studied. Chromogen or a substrate which changes color when cleaved by the enzyme is attached to the second antibody. ELISA allows for easy visualization of results and can be completed without the additional concern of radioactive materials use. (Johnson 1999).

Although the ELISA is a convenient and adequate procedure to quantify protein biomarkers using conventional sandwich immuno-assay with high sensitivity and specificity, it has some significant limitations. The assay requires high sample volume and cannot be multiplexed (multiple detection reactions carried out simultaneously in very small sample volumes). Muderhwa's strategy to optimize and validate radiation-

responsive protein biomarkers used a microsphere-based multi-analyte assay system (Luminex-100^{TM}). This technology was based on microscopic spherical polystyrol particles (microsphere) that serves as a solid phase for molecular detection reactions (McHugh, 1994) measured using a flow cytometer equipped with a 96-well micro-titer plate platform. The Luminex-100^{TM} system is capable of analyzing multiple assays (multi-analyte) simultaneously. Muderhwa's strategy used a mixture of two distinct sets of uniquely fluorescent micro-spheres, i.e., an array of fluorescent micro-spheres (Kettman *et al*., 1998), which were identified by distinct red and orange fluorescent internal dyes by the Luminex-100^{TM} flow analyzer (Fulton *et al*., 1997). At present, 100 distinct sets of fluorescent micro-spheres are available (See Figure 5). Quantification was accomplished with a green fluorescent reporter molecule. The light is read in a manner similar to optical density and reports a relative fluorescence. This fluorescence is what was used to find the data points in Figure 6. For this experiment, an increase in Luminex-100^{TM} fluorescence means an increase in GADD-45α response.

Furthermore, this technology demonstrates unsurpassed sensitivity, specificity, high-throughput potential and flexibility. The Luminex-100^{TM} system has been shown to be a feasible and cost-effective technology for assay development and multiplexing capability (Muderhwa, AFRRI intramural protocol 2003). Therefore by using the Luminex^{TM} Technology, this combines the benefits of rapid analysis, high-sample throughput, and definitive endpoint capabilities to assess radiation exposure in forward-deployable military laboratories and clinical settings. This is advantageous because it allows for multiple targets (GADD-45α, DDB-2, or BAX) to be looked at simultaneously and at a much shorter time when compared to running an ELISA for each target. On

average, the ELISA and the Luminex™ takes about the same time (eight-hours) to complete. However, if analyzing multiple targets was the goal, then the Luminex™ would save approximately eight-hours per target of interest.