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TITLE: Carcinogenicity and Immunotoxicity of Embedded Depleted Uranium and Heavy-Metal Tungsten Alloy in Rodents

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14. ABSTRACT This study investigates the carcinogenic and immunotoxic potential of embedded fragments of depleted uranium (DU) and a heavy-metal tungsten alloy (WA). Male Fisher 344 rats were surgically implanted with pellets of DU, WA, tantalum (inert metal, negative control), or nickel (known carcinogen, positive control). We previously reported that implanted WA resulted in the rapid formation of tumors, identified as rhabdomyosarcomas, surrounding the pellets. In Year 4 we found that these tumors had, within the same area, histopathological characteristics of both the pleomorphic and embryonal subtypes of rhabdomyosarcomas.					
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INTRODUCTION

Advancement in weapons design has led to the introduction of several potentially toxic metals, such as depleted uranium (DU), onto the battlefield. The Persian Gulf War in 1991 saw the first combat use of DU kinetic penetrator munitions, and their success against enemy armor was dramatic. The demonstrated effectiveness of DU munitions in the first Gulf War has led other nations, some not friendly to the United States, to adopt these weapons into their own arsenals. Other types of kinetic energy penetrators use heavy-metal tungsten alloys (WA) in place of DU. In future conflicts, the United States will have to deal with an increased number of casualties from the use of these weapons. Because both DU- and WA-based munitions are relatively recent additions to the list of militarily relevant metals, little is known about the health effects of these metals after internalization as embedded shrapnel. This study was designed to assess the carcinogenic and immunotoxic potential of DU and WA using the Fisher 344 rat model and modified National Toxicology Program protocols for such studies. Responses to the test metals are being compared to responses to tantalum, a biologically inert metal that serves as a negative control and nickel, a known heavy-metal toxin and carcinogen that serves as a positive control. This research addresses the DOD effort to understand the potential health risks associated with DU and WA exposure in order to develop appropriate medical treatment protocols for personnel wounded by fragments of these metals.

BODY

Statement of Work

This study is an assessment of the immunotoxic and carcinogenic potential of embedded fragments of DU and WA in laboratory rats. Responses to these metals are compared to the biologically inert metal, tantalum, and the carcinogen and heavy-metal toxin, nickel. For these experiments, rats are implanted with tantalum pellets alone (metal control group), a mixture of DU and tantalum pellets (low DU group), DU pellets alone (high DU group), a mixture of WA and tantalum pellets (low WA group), or WA pellets alone (high WA group). There is also a non-surgical control group and a positive carcinogenesis control group implanted with nickel pellets. Animals will be euthanized and various analyses performed 1, 3, 6, 12, 18, and 24 months after implantation. Analyses include histopathological examination and metal determinations as well as assessments of mutagenicity and cytogenicity. A battery of immunological tests designed to assess both humoral and cell-mediated immunity, as well as the innate immune response, will be conducted at 1, 3, 6, and 12 months.

Progress to Date

Because of technical and personnel issues, primarily concerning the metal measurement aspect of the project, we requested and received a one-year, no-cost extension of the project. Implantation surgery, euthanasia, and necropsy of all experimental groups are complete. General health parameters (body weight, hematological assessments, serum chemistries, etc.) and tumor incidence have been reported (Year 3 Annual Report). All immunotoxicity assessments have been completed. Serum and urine mutagenicity measurements have been completed. In Year 5, the primary task will be to finish the metal analysis of the experimental tissue samples and compile and statistically analyze that data. The data collected from the genotoxicity portion of the project will also be statistically analyzed. A timeline for the remaining Year 5 work is presented below, as are detailed results corresponding to the various project tasks.

Year 5 Schedule

- Compile cytogenetic data
- Complete metal measurements

Task 1 - *Determine whether embedded fragments of DU or WA cause cancer in rodents.*

Work was substantially completed and reported in the Year 3 Annual Report. In Year 4, further histopathological characterization of the WA-induced rhabdomyosarcomas was undertaken. There was little or no capsule formation around the WA pellet or associated tumors (Fig. 1 – Appendices). The tumor infiltrated into the skeletal muscle, separating and isolating individual myofibers. Eventually degeneration of the myofibers, with internalization of nuclei, was observed (Figs. 2 and 3 – Appendices). We found that cell pattern and morphology was variable, even within the same tumor (Fig. 4 – Appendices). Cell patterns ranged from distinct spindle cell in streams with strap-like cells to pleomorphic spindle cell pattern, to a round cell, spider

cell and giant cell patterns, more similar to embryonal rhabdomyosarcomas (Figs. 5-9 – Appendices).

The immunohistochemical analysis (Altmannsberger et al., 1985) of the WA-induced leg tumors, as well as the resulting metastatic lung tumors were reported in the Year 3 Annual Report and are also described in detail in our publication in *Environmental Health Perspectives* (Kalinich et al., 2005 - Appendices).

As also reported in the Year 3 report, rats implanted with 20 pellets of WA (high dose group) exhibited characteristics of polycythemia (elevated red blood cell counts, splenomegaly). Shown in Fig. 10 (Appendices) is a cross section of the spleen from one of these rats. There is a noticeable increase in nucleated red blood cells and a mild decrease in the myeloid:erythroid ratio in the red pulp indicative of erythroid hyperplasia.

No tumors, resulting from pellet implantation, were observed in the tantalum, low DU, or high DU groups at the 1-, 3-, 6-, 12-, 18-, or 24-month time points. Gross necropsies of these groups revealed no abnormalities. The 18- and 24-month animals, in all surviving groups, exhibited health problems associated with old age (e.g., testicular cancer, benign abdominal growths, etc.). These health problems were not associated with a particular treatment group. They were found across all experimental groups. As a result of these age-related health problems, many of the 18- and 24-month animals were euthanized before reaching their experimental endpoint; however, no abnormalities associated with the tantalum or DU implanted pellets were observed.

Task 2 - Measure tissue levels of DU or WA after chronic in vivo exposure.

During Year 4 we experienced significant technical problems with the inductively coupled plasma mass spectrometer (ICP-MS) used to conduct the metal measurements on our experimental samples. In addition, the individual responsible for operating this instrument is leaving the Institute. However, during Year 4, we completed the analytical preparation of the several thousand tissue samples collected in this project. Personnel associated with the grant were recently training in the operation and maintenance of the ICP-MS and have assumed the instrument operator's responsibilities. Shortly after completing training, we successfully resolved the issue of matrix effects (particularly in bone, liver, and kidney) that originally hindered our ability to accurately assess nickel and cobalt levels. Due to the extremely large number of samples to be analyzed (approximately 10000), this task will take the greater part of the next year, with the data to be presented in the final report.

Task 3 - Assess the genotoxicity and mutagenicity after chronic in vivo exposure to DU or WA.

All samples designated for genotoxicity assessment have been processed. The data is currently being compiled and statistically analyzed. Representative chromosome spreads, used for chromosomal analysis, are shown in Fig.11 of the Appendices. All genotoxicity data will be presented in the final report.

The mutagenicity assessment of serum samples from the 18-month experimental groups has been completed. There was no indication that serum from any of the experimental groups was mutagenic when assayed by the Ames bacterial reversion assay (Appendices - Table 1). Because the 24-month experimental groups were euthanized at

various times less than the full 24 months, no valid statistical comparison of the different experimental groups could be made concerning the mutagenic potential of that serum. As reported in the Year 3 Annual Report, no urine was collected from the 18- and 24-month animals because, due to the declining health of the rodents, we opted against stressing the animals further by housing them in the metabolic cages required for urine collection.

Task 4 - Determine the effect of embedded DU and WA on the organs of the immune system.

Substantially all the results pertaining to this task were presented in the Year 3 Annual Report, except for the flow cytometric analysis data which we are reporting here. Flow cytometric analysis of peripheral blood lymphocytes, splenocytes, and thymocytes was conducted in order to investigate any changes, as a result of prolonged exposure to embedded DU or WA, in the subpopulations of cells that comprise these immune system organs. Using the methods of Flaherty et al. (1997) and Capri et al. (2000), we determined the CD4 and CD8 subpopulations in isolated thymocytes; the cytotoxic T-cell, T-helper cell, and the naïve and activated subpopulation of CD4 and CD8 cells in peripheral blood; and the T cell, B cell, cytotoxic T cell, T helper cell, NK cell, and putative monocyte levels in isolated splenocytes (Tables 2-5 – Appendices).

For DU-implanted rats, the number of splenic NK cells were decreased 1 month post-implantation, but had returned to normal by 3 months post-implantation. All assessments at every other time point were not statistically different than control, with the exception of the 6-month splenic NK cells for the low-dose DU group which were significantly higher than control. However, at 12 months post-implantation there was no significant difference and the high-dose DU group showed no such changes.

For WA-implanted rats, there were numerous and consistent changes in immune cell subpopulations in both peripheral blood and spleen. High-dose WA-implanted rats showed significant decreases in peripheral blood cytotoxic T cells, T helper cells, and naïve CD4 and CD8 cells starting as early as 1 month post-implantation. Both the low- and high-dose WA rats demonstrated lower splenic B cell levels, as well as lower NK cell levels (as did both DU groups) at 1 month post-implantation. The peripheral blood subpopulation changes persisted in the high-dose WA rats at 3 months post-implantation. The splenic B cell levels returned to normal in both WA groups, but splenic NK cell levels remained depressed. By 6 months post-implantation, all WA rats had developed tumors at the pellet implantation site and were reaching the criteria for euthanasia. Not surprisingly, all peripheral blood flow cytometric parameters were significantly lower in both the low- and high-WA animals as compared to control. Somewhat surprisingly, values for the various immune system cell subpopulations in the spleen were not statistically different from control, except for splenic NK cell levels which had risen significantly higher. No WA animal survived to the 12 month time point.

Interestingly, there were no significant changes in CD4/CD8 thymocyte subpopulations between the various treatment groups at any of the time points assayed. Also, due to a computer hard drive problem, the peripheral blood and splenic flow cytometric analysis for non-surgical 6-month animals was lost. For this time point, statistical comparison was done using the tantalum control animals as the “control” group. There were no statistical differences between the non-surgical group and the tantalum control group at any time point for any of the parameters assessed, except for 1

month splenic NK cell levels. Since this was observed in all implantation groups, we believe it is a result of the surgery itself. However, the tantalum control levels, although statistically lower than the non-surgical control levels, were still significantly higher than the levels found in both DU and WA groups. This may be the result of toxicity of the metals specific to those implants.

Task 5 – *Evaluate the effect chronic in vivo exposure to DU and WA has on immune function, including cell-mediated, humoral, and innate immunity.*

This task was completed and results reported in the Year 3 annual report.

KEY RESEARCH ACCOMPLISHMENTS

- All rodents have been implanted and have reached their experimental endpoints.
- All animals implanted with WA (both low and high groups) developed aggressive rhabdomyosarcomas at the implantation site and the tumors eventually metastasized to the lung.
- Histopathological examination showed that the tumors exhibited characteristics of both classic or pleomorphic rhabdomyosarcomas as well as embryonal rhabdomyosarcomas.
- Rodents in the high WA group exhibited altered distribution of lymphocyte subpopulations in both peripheral blood and spleen as early as one month after pellet implantation.
- No effect of pellet implantation on CD4/CD8 thymocyte subpopulations was observed for any of the experimental groups.

REPORTABLE OUTCOMES

Oral presentations

Oral presentation by Dr. John F. Kalinich at the TSCA ITC Meeting, Washington, DC, May 5, 2005. Title: Health effects of tungsten alloys.

Oral presentation by Dr. David McClain at the NATO RTG-099 Meeting, Bethesda, MD, June 21-23, 2005. Title: Status of health concerns about military use of depleted uranium and surrogate metals in armor penetrating munitions.

Oral presentation by Dr. David McClain at the 8th Annual Force Health Protection Conference, Louisville, KY, August 7-12, 2005. Title: Embedded tungsten alloy fragment research and its implications.

Oral presentation by Dr. John Kalinich at the American Chemical Society 230th National Meeting, Washington, DC, August 28 – September 1, 2005. Title: Embedded weapons-grade tungsten alloy shrapnel rapidly induces metastatic high-grade rhabdomyosarcomas in F344 rats.

Abstract

David E. McClain and John F. Kalinich, Embedded weapons-grade tungsten alloy shrapnel rapidly induces metastatic high-grade rhabdomyosarcomas in F344 rats, International Conference on Tungsten, Refractory and Hardmetals.

Manuscript

John F. Kalinich, Christy A. Emond, Thomas K. Dalton, Steven R. Mog, Gary D. Coleman, Jessica E. Kordell, Alexandra C. Miller, and David E. McClain, Embedded weapons-grade tungsten alloy shrapnel rapidly induces metastatic high-grade rhabdomyosarcomas in F344 rats, *Environmental Health Perspectives* **113**: 729-734 (2005).

Two additional manuscripts are in preparation.

CONCLUSIONS

The project encountered some delays this year when we experienced significant technical issues with our inductively coupled plasma mass spectrometer (ICP-MS), used for metal analysis of our tissue samples. In addition, we lost two key personnel involved in the project to retirement. As a result, we requested a one-year, no-cost extension for the project, which was granted (Letter – Appendices). During this year we have had several additional personnel trained in the operation and maintenance of the ICP-MS and they have assumed the departing instrument operator's responsibilities. Metal analysis of the several thousand tissue samples collected is the most important task remaining. This year, all samples were prepared for analysis and several technical issues with the analytical procedure successfully resolved. Metal analysis is currently underway and no major issues have been encountered by the recently trained operators. We expect that it will take 3 to 4 months to assay all of the samples, with additional time required for data compilation and statistical analysis. We expect that all work will be completed well before the one-year extension ends. The only other remaining work involves compilation and analysis of our genotoxicity data. We will also continue our histopathological analysis of experimental tissues. We plan to store these tissues in order that, in the future, additional testing can occur as technical methods become more advanced.

As noted earlier, all experimental animals have been implanted and reached their euthanasia time points. No pellet-associated tumors were found in tantalum- or DU-implanted animals. However, rapid and aggressive tumor development occurred at the implantation sites in the WA groups. This raises significant concern since heavy-metal tungsten alloys are being used as replacements for DU in armor penetrators. In fact, many countries, some unfriendly to the U.S., already possess tungsten munitions. As a result, future combat could produce large numbers of U.S. personnel with tungsten fragment injuries with military surgeons not having the best information available to deal with those injuries.

All immunotoxicity assessments have been completed and reported. The majority of the data were reported last year. This year we have shown the high-dose WA-implanted rats exhibited significant alterations in the distribution of lymphocyte

subpopulations in both peripheral blood and spleen as early as one month after pellet implantation. The perturbations continued throughout the life of the animal. Low-dose WA-implanted animals showed the same effects later in life, indicating a potential deleterious effect on the immune system with increasing amounts of embedded WA.

It should be pointed out that the tungsten alloy we used consisted of tungsten, nickel, and cobalt. Other tungsten alloys, tungsten/nickel/iron in particular, are also being used to produce armor penetrating munitions. Because of the lack of information on the health effects of embedded fragments composed of these types of mixtures, we strongly recommend funding be made immediately available for an additional study to investigate potential tumor development patterns for implanted tungsten/nickel/iron, as well as for the individual metals alone and in various combinations. With these data, a determination of the carcinogenic potential of the individual metals can be made, as well as a determination of any synergistic effects that may occur when the various metal are present together. With these results, a comparison of the various alloys can be made and the exposure risk put into perspective.

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APPENDICES

Figure 1 - Masson Trichrome Stain of WA-Induced Tumor
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Manuscript - Environmental Health Perspectives

FIGURE 1. Masson Trichrome Stain of WA-Induced Tumor

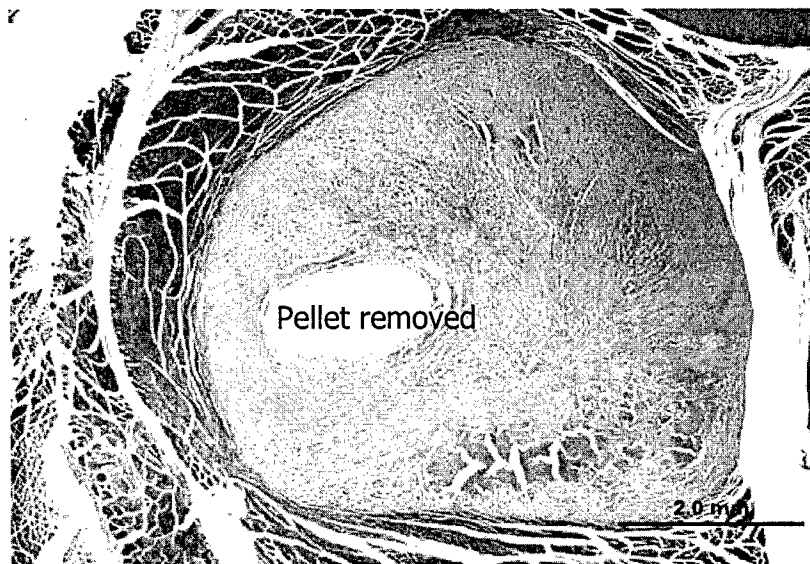


FIGURE 2. Sarcoma Infiltration into Skeletal Muscle.

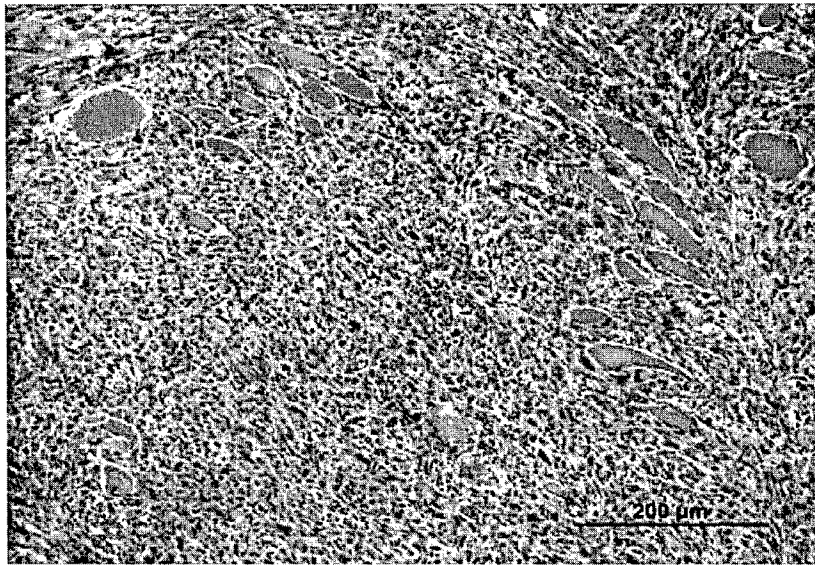


FIGURE 3. Sarcoma-Induced Muscle Degeneration

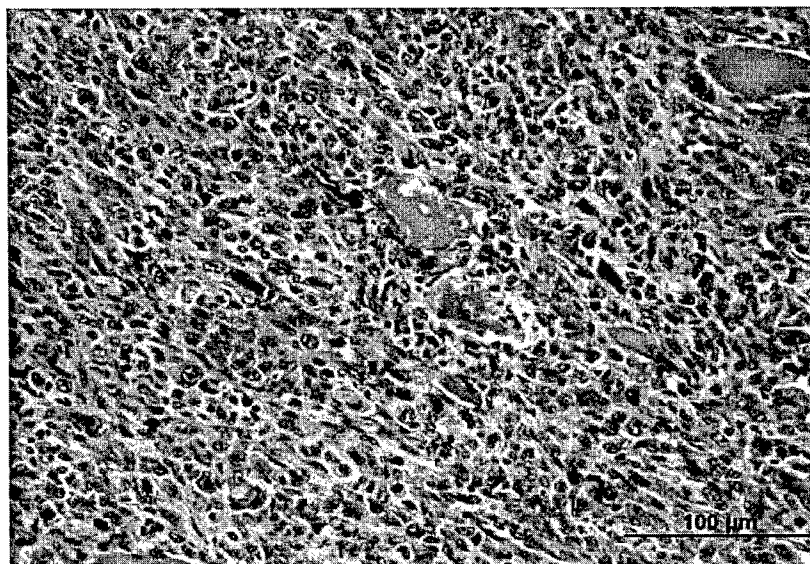


FIGURE 4. Variable Pattern of WA-Induced Rhabdomyosarcoma Showing Classic or Pleomorphic Rhabdomyosarcoma (lower left corner) and Embryonal Rhabdomyosarcoma (upper right corner)

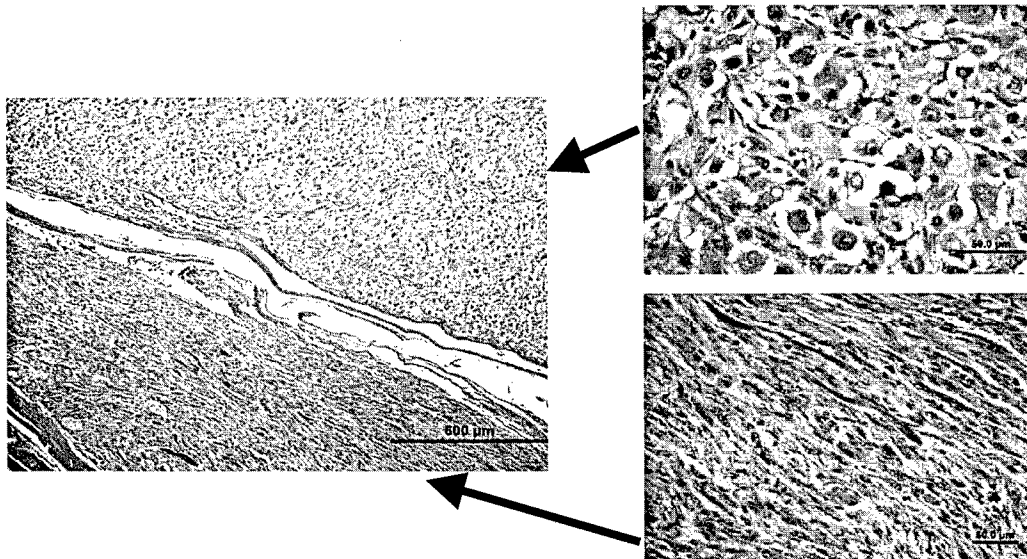


FIGURE 5. Neoplastic Spindle Cells and Giant Neoplastic Cells

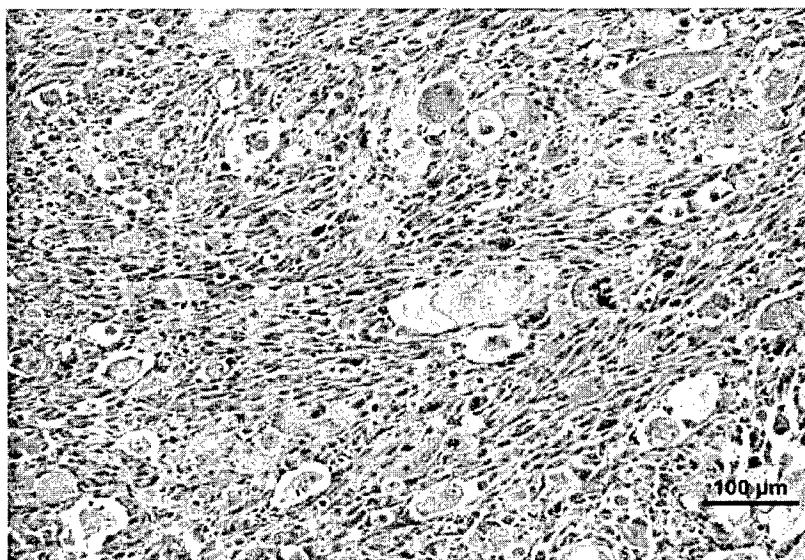


FIGURE 6. Pleomorphic Cells

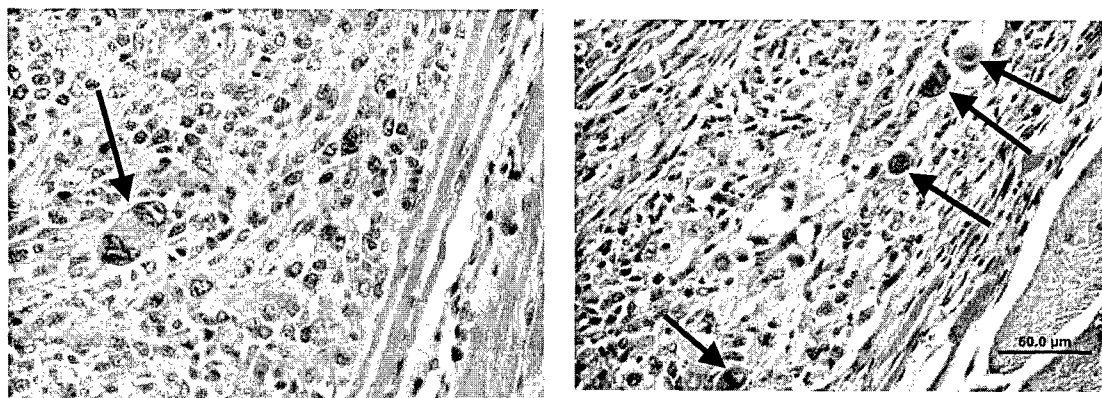


FIGURE 7. Vacuolated Spider Cells

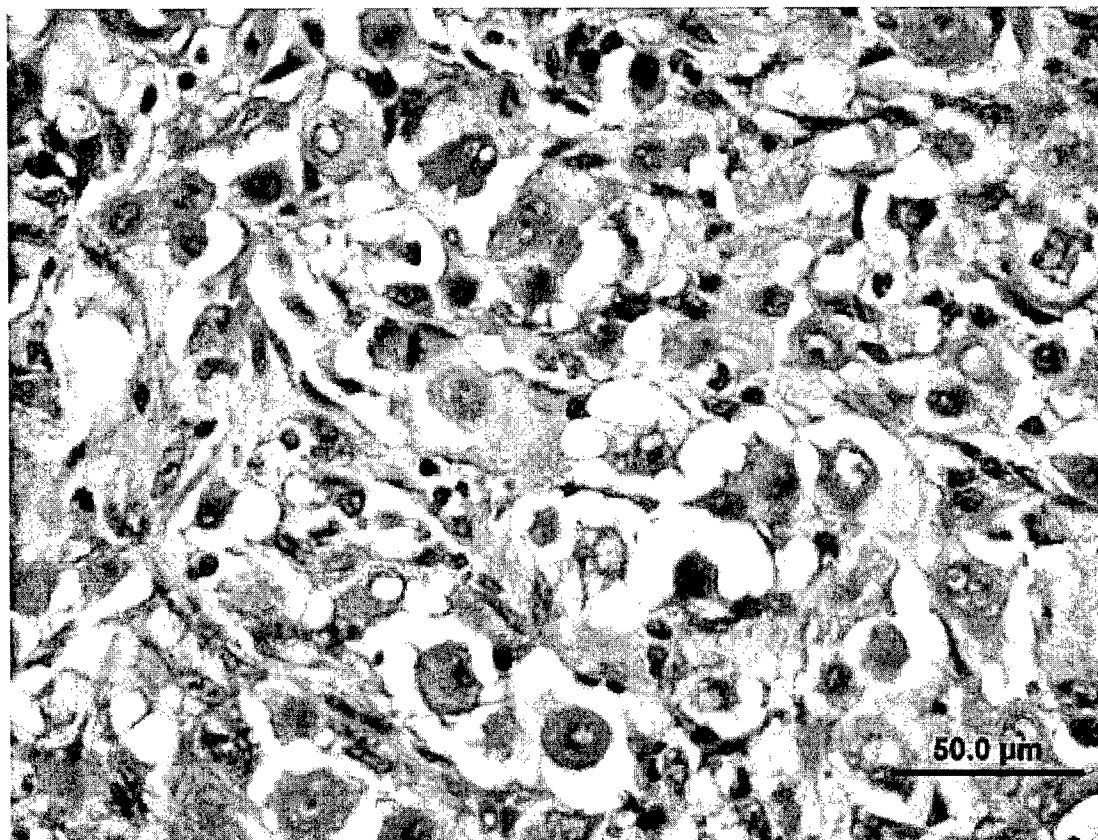


FIGURE 8. Multinucleate Giant Cells

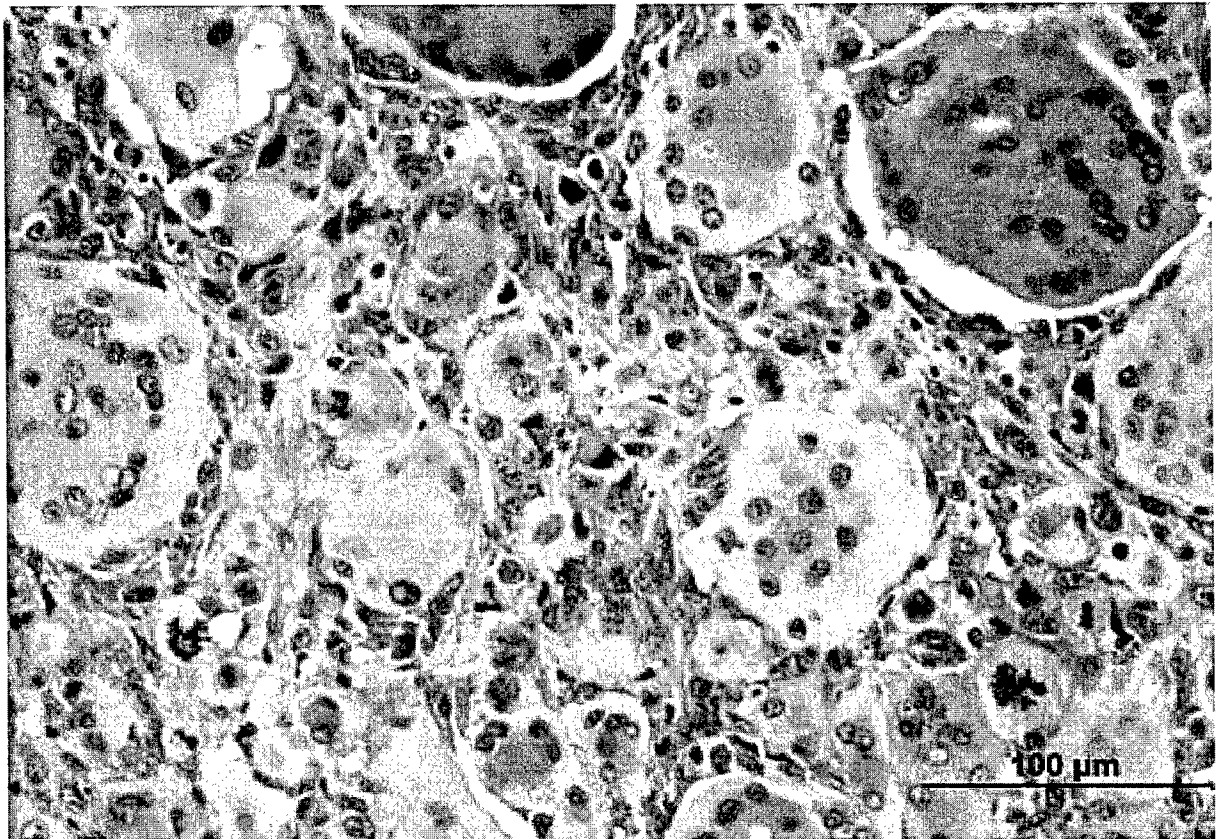


FIGURE 9. Multinucleate Giant and Strap Cells.

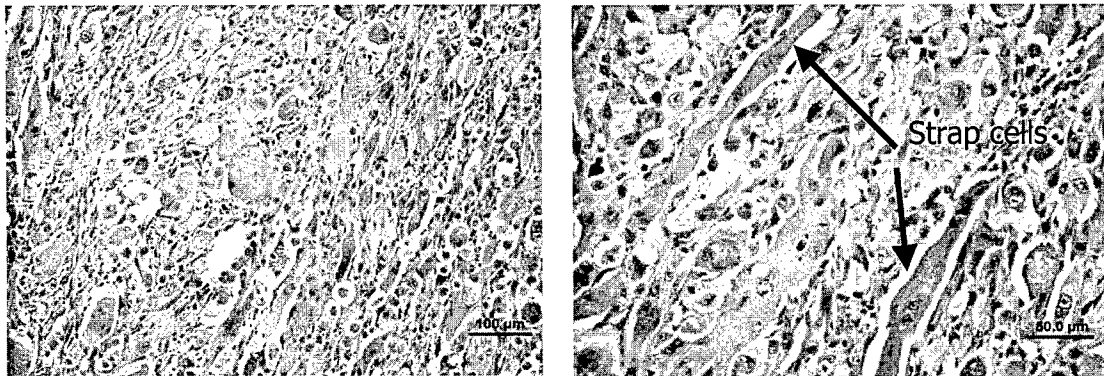


FIGURE 10. Splenic Red Pulp



FIGURE 11. Metaphase Chromosome Spread from Rat Lymphocytes

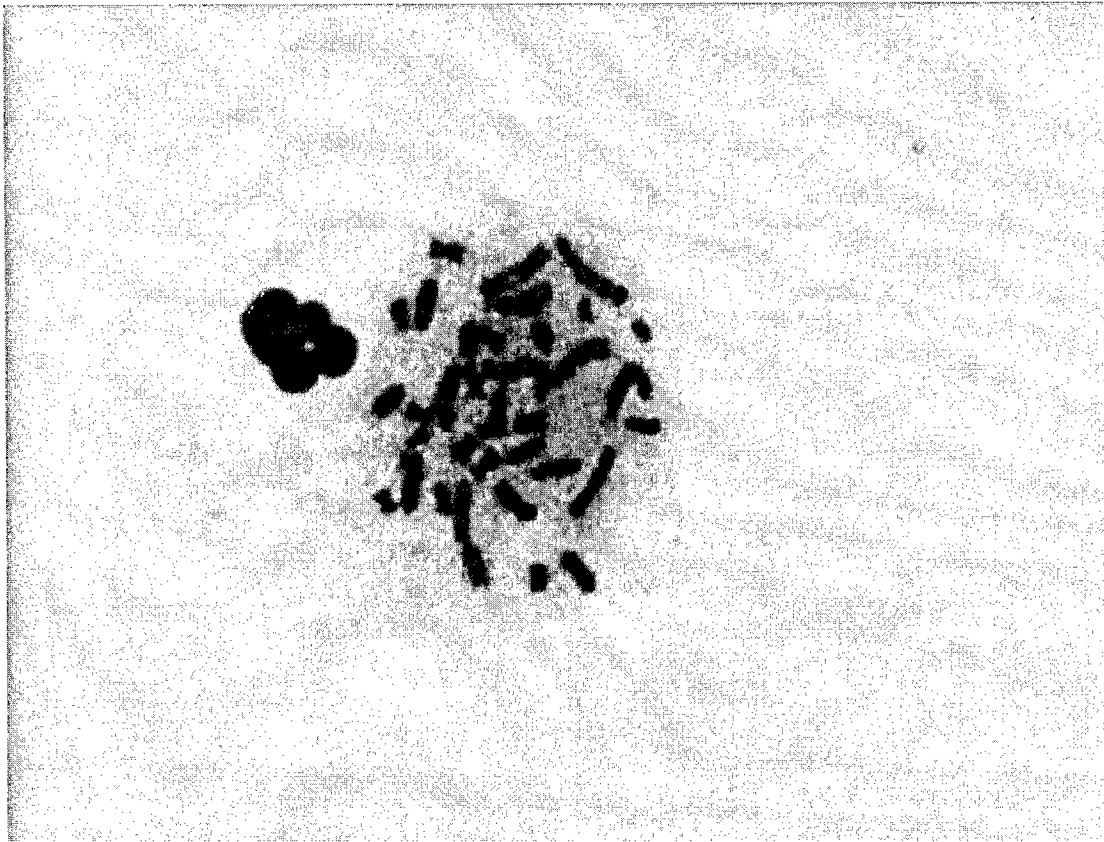


TABLE 1

Mutagenicity of Serum Samples from 18-Month Experimental Animals

Experimental Group	Mutagenicity (revertants/μl serum)
Non-surgical (NS)	0.195 ± 0.024
Tantalum (Ta)	0.159 ± 0.032
DU-low (DU-L)	0.188 ± 0.049
DU-high (DU-H)	0.121 ± 0.088

Data are represented as the mean of 3 to 9 observations (NS: n=7; Ta: n=9; DU-L: n=3; DU-H: n=5) with error reported as standard deviation. Results were not significantly different (at $P < 0.05$) from non-surgical control group using Students t-test.

TABLE 2. Flow Cytometric Analysis – 1 Month Animals

Thymus						
Group	CD4-CD8-	CD4+CD8-	CD4-CD8+	CD4+CD8+		
NS	3.32 ± 0.14	6.68 ± 0.31	4.70 ± 0.09	85.30 ± 0.43		
TC	4.04 ± 0.29	6.92 ± 0.28	4.55 ± 0.32	84.50 ± 0.72		
DL	3.05 ± 0.13	5.77 ± 0.42	4.17 ± 0.13	87.01 ± 0.41		
DH	3.59 ± 0.15	6.17 ± 0.21	4.46 ± 0.14	85.78 ± 0.39		
WL	3.32 ± 0.12	6.14 ± 0.18	4.39 ± 0.19	86.15 ± 0.31		
WH	3.31 ± 0.10	5.46 ± 0.20	4.66 ± 0.10	86.56 ± 0.28		

Blood						
Group	Cytotoxic T cell	T helper	Naïve CD4	Activated CD4	Naïve CD8	Activated CD8
NS	11.14 ± 1.19	18.61 ± 2.04	13.15 ± 1.46	6.30 ± 0.75	13.50 ± 1.95	3.24 ± 1.08
TC	12.71 ± 0.95	21.53 ± 1.50	13.66 ± 0.91	8.76 ± 0.65	12.09 ± 1.16	1.01 ± 0.07
DL	10.57 ± 0.61	18.16 ± 0.88	9.70 ± 0.42	9.22 ± 0.68	9.94 ± 0.55	1.07 ± 0.09
DH	11.04 ± 1.09	18.22 ± 1.98	10.10 ± 1.03	7.12 ± 0.77	9.37 ± 0.74	1.11 ± 0.15
WL	10.52 ± 0.74	17.29 ± 1.15	10.14 ± 1.08	8.48 ± 0.59	9.71 ± 0.81	1.19 ± 0.19
WH	7.50 ± 0.39 *	12.75 ± 0.76 *	5.75 ± 0.70 *	6.98 ± 1.03	6.74 ± 0.35 *	0.90 ± 0.07

Spleen						
Group	T cells	B cells	Cytotoxic T cells	NK cells	T helper	Putative Monocytes
NS	27.26 ± 0.45	45.30 ± 0.64	9.46 ± 0.33	9.32 ± 0.25	12.88 ± 0.19	4.55 ± 0.25
TC	25.45 ± 0.45	46.77 ± 0.99	18.30 ± 0.56	6.75 ± 0.56 *	11.26 ± 0.31	3.24 ± 0.19
DL	25.47 ± 0.32	43.77 ± 1.83	17.55 ± 0.37	2.29 ± 0.18 *	11.12 ± 0.29	3.33 ± 0.22
DH	26.55 ± 0.79	42.41 ± 1.19	18.31 ± 0.49	4.73 ± 0.39 *	11.78 ± 0.18	3.87 ± 0.19
WL	25.98 ± 0.44	41.05 ± 0.65 *	18.74 ± 0.50	4.64 ± 0.41 *	11.70 ± 0.32	3.90 ± 0.13
WH	25.90 ± 0.50	39.10 ± 0.59 *	18.30 ± 0.27	3.87 ± 0.26 *	12.00 ± 0.24	3.25 ± 0.11

* Statistically different from control using one-way ANOVA followed by Dunnett's test for group comparisons ($P < 0.05$). Groups: NS (Non-surgical); TC (Tantalum control); DL (DU-low dose); DH (DU-high dose); WL (WA-low dose); WH (WA-high dose).

TABLE 3. Flow Cytometric Analysis – 3 Month Animals

Thymus						
<u>Group</u>	<u>CD4-CD8-</u>	<u>CD4+CD8-</u>	<u>CD4-CD8+</u>	<u>CD4+CD8+</u>		
NS	4.31 ± 0.42	7.10 ± 0.44	4.71 ± 0.24	83.88 ± 1.06		
TC	3.69 ± 0.10	6.59 ± 0.18	4.65 ± 0.12	85.07 ± 0.29		
DL	3.47 ± 0.14	6.39 ± 0.17	4.72 ± 0.18	85.43 ± 0.36		
DH	3.54 ± 0.23	6.25 ± 0.36	4.55 ± 0.34	85.66 ± 0.47		
WL	3.78 ± 0.19	7.04 ± 0.22	4.30 ± 0.17	84.88 ± 0.49		
WH	3.42 ± 0.09	6.50 ± 0.22	3.95 ± 0.12	86.13 ± 0.20		

Blood						
<u>Group</u>	<u>Cytotoxic T cell</u>	<u>T helper</u>	<u>Naïve CD4</u>	<u>Activated CD4</u>	<u>Naïve CD8</u>	<u>Activated CD8</u>
NS	12.72 ± 1.07	20.84 ± 1.59	12.67 ± 1.20	8.83 ± 0.67	12.33 ± 1.13	1.57 ± 0.11
TC	11.67 ± 1.40	18.52 ± 2.19	11.76 ± 1.42	8.50 ± 0.90	11.16 ± 1.26	0.90 ± 0.16
DL	13.12 ± 1.14	20.64 ± 1.70	12.37 ± 1.13	8.50 ± 0.85	12.52 ± 1.13	1.29 ± 0.13
DH	11.74 ± 0.64	18.66 ± 1.02	7.01 ± 1.31	5.54 ± 1.30	10.92 ± 0.59	1.10 ± 0.08
WL	13.19 ± 0.83	20.50 ± 1.34	11.48 ± 0.76	9.43 ± 0.71	12.34 ± 0.78	1.33 ± 0.10
WH	7.86 ± 0.75 *	13.54 ± 0.80 *	7.44 ± 0.52 *	6.44 ± 0.47 *	8.03 ± 0.58 *	0.94 ± 0.10

Spleen						
<u>Group</u>	<u>T cells</u>	<u>B cells</u>	<u>Cytotoxic T cells</u>	<u>NK cells</u>	<u>T helper</u>	<u>Putative Monocytes</u>
NS	26.58 ± 0.59	44.58 ± 0.76	20.24 ± 0.41	6.58 ± 0.55	11.69 ± 0.22	3.27 ± 0.10
TC	26.79 ± 0.54	46.13 ± 0.71	20.77 ± 0.37	6.01 ± 0.34	11.99 ± 0.23	2.94 ± 0.11
DL	27.16 ± 0.70	41.09 ± 3.08	20.70 ± 0.45	4.85 ± 0.35	12.69 ± 0.29	3.11 ± 0.15
DH	27.78 ± 0.70	43.79 ± 1.06	21.81 ± 0.46	6.38 ± 0.34	12.43 ± 0.26	3.38 ± 0.33
WL	26.32 ± 0.61	43.37 ± 0.65	19.13 ± 0.46	4.31 ± 0.24 *	11.64 ± 0.19	3.40 ± 0.14
WH	25.50 ± 0.45	43.54 ± 0.72	18.86 ± 0.46	4.69 ± 0.32 *	10.97 ± 0.29	3.15 ± 0.16

* Statistically different from control using one-way ANOVA followed by Dunnett's test for group comparisons ($P < 0.05$). Groups: NS (Non-surgical); TC (Tantalum control); DL (DU-low dose); DH (DU-high dose); WL (WA-low dose); WH (WA-high dose).

TABLE 4. Flow Cytometric Analysis – 6 Month Animals

Thymus						
Group	CD4-CD8-	CD4+CD8-	CD4-CD8+	CD4+CD8+		
NS	6.14 ± 0.42	8.16 ± 0.34	5.01 ± 0.18	81.51 ± 0.78		
TC	6.35 ± 0.20	9.23 ± 0.25	5.28 ± 0.12	79.14 ± 0.50		
DL	7.31 ± 0.40	8.81 ± 0.68	5.95 ± 0.32	77.93 ± 1.02		
DH	4.78 ± 0.44	6.74 ± 0.28	4.81 ± 0.24	83.67 ± 0.85		
WL	4.86 ± 0.32	6.85 ± 0.19	4.67 ± 0.17	83.62 ± 0.56		
WH	4.41 ± 0.22	6.75 ± 0.28	4.31 ± 0.11	84.54 ± 0.54		

Blood						
Group	Cytotoxic T cell	T helper	Naïve CD4	Activated CD4	Naïve CD8	Activated CD8
NS	ND	ND	ND	ND	ND	ND
TC	10.69 ± 1.23	17.18 ± 1.85	10.72 ± 1.32	7.17 ± 0.77	10.24 ± 1.21	1.23 ± 0.14
DL	9.88 ± 0.51	15.45 ± 1.07	10.66 ± 0.37	6.46 ± 1.07	9.94 ± 0.61	0.99 ± 0.09
DH	10.66 ± 1.06	16.36 ± 1.58	10.06 ± 0.92	7.03 ± 0.87	10.04 ± 1.01	1.29 ± 0.19
WL	6.07 ± 1.24 *	9.50 ± 2.27 *	5.12 ± 1.68 *	4.64 ± 1.09 *	5.45 ± 1.11 *	1.12 ± 0.56
WH	5.41 ± 0.89 *	8.15 ± 1.40 *	4.60 ± 0.77 *	3.76 ± 0.66 *	5.14 ± 0.93 *	0.54 ± 0.12 *

Spleen						
Group	T cells	B cells	Cytotoxic T cells	NK cells	T helper	Putative Monocytes
NS	ND	ND	ND	ND	ND	ND
TC	26.69 ± 0.56	45.35 ± 0.72	20.51 ± 0.39	6.30 ± 0.44	11.84 ± 0.23	3.17 ± 0.10
DL	24.07 ± 0.44	45.63 ± 1.10	21.96 ± 0.79	9.70 ± 0.31 *	12.25 ± 0.39	4.78 ± 0.42
DH	26.65 ± 0.32	43.39 ± 1.10	21.12 ± 0.50	6.41 ± 0.56	13.39 ± 0.51	3.36 ± 0.14
WL	23.97 ± 1.00	42.76 ± 1.49	21.57 ± 1.44	9.13 ± 0.65 *	13.00 ± 0.80	4.33 ± 0.34
WH	23.69 ± 0.53 *	44.64 ± 0.71	19.61 ± 0.42	10.25 ± 0.46 *	10.91 ± 0.15	3.25 ± 0.10

* Statistically different from control using one-way ANOVA followed by Dunnett's test for group comparisons ($P < 0.05$). Groups: NS (Non-surgical); TC (Tantalum control); DL (DU-low dose); DH (DU-high dose); WL (WA-low dose); WH (WA-high dose). ND – not determined.

TABLE 5. Flow Cytometric Analysis – 12 Month Animals

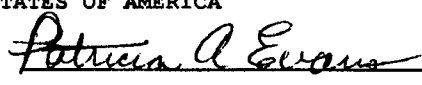
Thymus						
Group	CD4-CD8-	CD4+CD8-	CD4-CD8+	CD4+CD8+		
NS	18.04 ± 2.37	14.66 ± 1.67	7.96 ± 0.43	59.34 ± 4.22		
TC	21.33 ± 3.50	14.02 ± 1.27	8.68 ± 0.77	55.98 ± 5.36		
DL	17.98 ± 2.07	13.47 ± 1.28	8.48 ± 0.50	60.07 ± 3.75		
DH	19.38 ± 2.46	15.06 ± 1.58	8.40 ± 0.66	57.16 ± 4.63		

Blood						
Group	Cytotoxic T cell	T helper	Naïve CD4	Activated CD4	Naïve CD8	Activated CD8
NS	3.82 ± 0.48	6.59 ± 0.90	3.05 ± 0.53	3.82 ± 0.57	3.58 ± 0.50	0.73 ± 0.11
TC	4.53 ± 0.72	7.59 ± 1.17	3.07 ± 0.52	4.40 ± 0.61	3.98 ± 0.61	0.72 ± 0.14
DL	5.14 ± 1.00	8.12 ± 1.43	3.51 ± 0.69	4.97 ± 0.84	4.49 ± 0.87	0.91 ± 0.18
DH	5.90 ± 0.93	9.08 ± 1.36	4.49 ± 0.71	4.89 ± 0.72	5.26 ± 0.82	0.88 ± 0.15

Spleen						
Group	T cells	B cells	Cytotoxic T cells	NK cells	T helper	Putative Monocytes
NS	23.11 ± 0.33	43.27 ± 0.79	22.34 ± 0.63	10.75 ± 0.93	10.52 ± 0.32	5.28 ± 0.61
TC	21.23 ± 0.57	40.95 ± 1.72	21.11 ± 0.31	11.47 ± 0.94	9.85 ± 0.49	7.60 ± 1.10
DL	22.11 ± 0.49	44.82 ± 0.62	21.22 ± 0.51	11.06 ± 0.65	11.12 ± 0.26	5.28 ± 0.54
DH	22.64 ± 0.44	43.21 ± 1.03	22.30 ± 0.54	11.76 ± 0.82	11.30 ± 0.35	5.61 ± 0.67

* Statistically different from control using one-way ANOVA followed by Dunnett's test for group comparisons ($P < 0.05$). Groups: NS (Non-surgical); TC (Tantalum control); DL (DU-low dose); DH (DU-high dose).

ASSISTANCE AGREEMENT

AWARD TYPE: ☒ GRANT (31 USC 6304) ☐ COOPERATIVE AGREEMENT (31 USC 6305) ☐ OTHER TRANSACTION (10 USC 2371)			
AWARD NO: DAMD17-01-1-0821 Modification No. P00002		EFFECTIVE DATE See Grants Officer Signature Date Below	
		AWARD AMOUNT \$983,164.00	
		Page 1 of Steve Rice, 301-619-8801 (phone) 301-619-4084 (fax)	
PROJECT TITLE: Carcinogenicity and Immunotoxicity of Embedded Depleted Uranium and Heavy-Metal Tungsten Alloy in Rodents CFDA 12.420			
PERFORMANCE PERIOD: 17 Sept. 2001 - 16 Oct. 2006 (Research ends 16 Sept. 2006)		PRINCIPAL INVESTIGATOR: Dr. John F. Kalinich	
AWARDED AND ADMINISTERED BY: U.S. Army Medical Research Acquisition Activity ATTN: MCMR-AAA-R 820 Chandler St. Fort Detrick Maryland 21702-5014		PAYMENTS WILL BE MADE BY: EFT:T Army Vendor Pay DFAS-SA-FPA 500 McCullough Avenue San Antonio, Tx 78215-2100	
DUNS No: 14-467-6566 TIN No:		(SEE PARAGRAPH TITLED "PAYMENTS" FOR INSTRUCTIONS)	
AWARDED TO: Henry M. Jackson Foundation for the Advancement of Military Medicine 1401 Rockville Pike, Suite 600 Rockville, MD 20852		REMIT PAYMENT TO: See "Awarded To" Block	
ACCOUNTING AND APPROPRIATION DATA:			
SCOPE OF WORK: IDEA Award In accordance with the Assistance Agreement, Page4, Paragraph c. and the recipient's e-mail dated July 21, 2005, which is incorporated herein by reference, the period of performance is changed as follows: From: 17 September 2001 - 16 October 2005 (Research ends 16 September 2005) To: 17 September 2001 - 16 October 2006 (Research ends 16 September 2006) There are no other changes to this Assistance Agreement.			
RECIPIENT		GRANTS OFFICER	
ACCEPTED BY: Signature contained on recipient's e-mail dated July 21, 2005. 		UNITED STATES OF AMERICA  _____ SIGNATURE	
SIGNATURE		SIGNATURE	
NAME AND TITLE	DATE	NAME AND TITLE	DATE
		PATRICIA A. EVANS GRANTS OFFICER	23 Aug 05

Embedded Weapons-Grade Tungsten Alloy Shrapnel Rapidly Induces Metastatic High-Grade Rhabdomyosarcomas in F344 Rats

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Continuing concern regarding the potential health and environmental effects of depleted uranium and lead has resulted in many countries adding tungsten alloy (WA)-based munitions to their battlefield arsenals as replacements for these metals. Because the alloys used in many munitions are relatively recent additions to the list of militarily relevant metals, very little is known about the health effects of these metals after internalization as embedded shrapnel. Previous work in this laboratory developed a rodent model system that mimicked shrapnel loads seen in wounded personnel from the 1991 Persian Gulf War. In the present study, we used that system and male F344 rats, implanted intramuscularly with pellets (1 mm × 2 mm cylinders) of weapons-grade WA, to simulate shrapnel wounds. Rats were implanted with 4 (low dose) or 20 pellets (high dose) of WA. Tantalum (20 pellets) and nickel (20 pellets) served as negative and positive controls, respectively. The high-dose WA-implanted rats ($n = 46$) developed extremely aggressive tumors surrounding the pellets within 4–5 months after implantation. The low-dose WA-implanted rats ($n = 46$) and nickel-implanted rats ($n = 36$) also developed tumors surrounding the pellets but at a slower rate. Rats implanted with tantalum ($n = 46$), an inert control metal, did not develop tumors. Tumor yield was 100% in both the low- and high-dose WA groups. The tumors, characterized as high-grade pleomorphic rhabdomyosarcomas by histopathology and immunohistochemical examination, rapidly metastasized to the lung and necessitated euthanasia of the animal. Significant hematologic changes, indicative of polycythemia, were also observed in the high-dose WA-implanted rats. These changes were apparent as early as 1 month postimplantation in the high-dose WA rats, well before any overt signs of tumor development. These results point out the need for further studies investigating the health effects of tungsten and tungsten-based alloys. **Key words:** cobalt, embedded fragment, nickel, rat, rhabdomyosarcoma, tungsten, tungsten alloy. *Environ Health Perspect* 113:729–734 (2005). doi:10.1289/ehp.7791 available via <http://dx.doi.org/> [Online 15 February 2005]

Tungsten has been used for many years in a variety of applications. Combining the hard, brittle tungsten metal with various other metals, including nickel and cobalt, produces tungsten alloys (WAs) with specific characteristics, some of which are of interest to the military. Recently, WAs have replaced lead in some small-caliber ammunition (the “green bullet”) [Oak Ridge National Laboratory (ORNL) 1998] and depleted uranium (DU) in kinetic-energy penetrators (ORNL 1996). Based on a small number of studies, prevailing theory is that elemental tungsten or insoluble tungsten compounds have only limited toxicity (Leggett 1997). For example, tungsten coils implanted into the subclavian artery of rabbits rapidly degrade, leading to elevated serum tungsten levels as early as 15 min after implantation. However, after 4 months, no signs of local or systemic toxicity were observed (Peuster et al. 2003). Studies on health effects of Ni and Co are more numerous. Intramuscular injections (28 mg) of soluble metallic Ni or Co result in formation of rhabdomyosarcomas at the injection site. With Ni, 100% of injected rats develop a tumor within 41 weeks (Heath and Daniel 1964), whereas administration of Co

results in tumor formation in 40% of the rats with a latency period of 71 weeks (Heath 1954, 1956). However, intramuscular implantation of rods or pellets composed of various Ni or Co alloys used in orthopedic prosthetics results in no excessive tumor formation (Gaechter et al. 1977; Sunderman 1989). A variety of other Ni compounds, including nickel subsulfide, nickel oxide, and nickel monosulfide, have been tested for carcinogenic potential via intramuscular administration (Gilman 1962; Sunderman and Maenza 1976; Sunderman et al. 1977). Tumors (rhabdomyosarcoma and fibrosarcoma) were found in many cases at the injection site, with tumor yield dependent on solubility and concentration of the administered compound. It has been postulated that the yield of localized tumors is inversely related to the rate of solubilization of the Ni-containing compound (Kasprzak et al. 1983). This hypothesis does not appear to hold for Co compounds (Lison et al. 2001).

Metal alloys present additional problems when investigating health effects. The various metals comprising the alloy, as well as the method of production, can all factor into the overall health effect observed upon exposure.

Investigations on hard-metal disease have shown that either tungsten carbide or Co alone has limited toxicity on lung tissue (Lasfargues et al. 1992). However, when combined, the tungsten carbide/cobalt mixture acts synergistically to increase the observed toxicity. It is not known whether this is due to the combined toxicity of the tungsten carbide/cobalt mixture or to an increase in the bioavailability of the known toxicant, Co (Lison and Lauwerys 1997). *In vitro* studies investigating malignant transformation of immortalized human cells by mixtures of tungsten, Ni, and Co suggest a synergistic effect that greatly exceeds the effects of the metals individually (Miller et al. 2001, 2002).

Advancements in metallurgy have led the military of many nations to replace DU in some armor-penetrating munitions and lead in small-caliber ammunition with various alloys of tungsten. One motivation for such a replacement is widespread public concern about the health and environmental impact of continued use of these metals. However, to our knowledge, none of these militarily relevant WAs has been tested for potential health effects, especially as embedded shrapnel. There is a growing list of health concerns related to tungsten exposure. Although a definitive link has not been established, several cancer clusters in the United States are associated with elevated levels of tungsten in the environment. Those findings, along with the results presented in this article, raise questions about the possible consequences of tungsten exposure. More important, these results raise extremely serious concerns over the potential health effects of WA-based munitions currently being used as nontoxic alternatives to lead and DU.

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The views and opinions expressed in this report are strictly those of the authors and should not be construed as official U.S. Department of Defense policy.

The authors declare they have no competing financial interests.

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Materials and Methods

Rodents. Male F344 rats (6 weeks of age; Harlan, Frederick, MD) were maintained in a facility accredited by the Association of Assessment and Accreditation of Laboratory Animal Care in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996). All procedures, including euthanasia criteria (Tomasovic et al. 1988), were approved by the Armed Forces Radiobiology Research Institute's (AFRRI) Animal Care and Use Committee. Upon arrival, animals were screened for common rodent pathogens. Rats were pair-housed in plastic microisolator cages with hardwood chips for bedding and fed a certified NTP-2000 (Quality Lab Products, Elkridge, MD) diet (Rao 1996) with acidified water provided *ad libitum*. Animals were on a 12-hr light/dark cycle with no twilight and were weighed weekly.

Pellets. All metal pellets were cylinders 1 mm in diameter and 2 mm in length. Nickel (99.995% metallic Ni) and tantalum (99.95% Ta) pellets were purchased from Alfa Aesar (Ward Hill, MA). WA pellets were fabricated by Aerojet Ordnance Tennessee (Jonesborough, TN) using standard kinetic energy penetrator production processes. An average WA pellet weighed 27.5 mg and consisted of 91.1% tungsten, 6.0% Ni, and 2.9% Co. Ni and Ta pellets weighed 14 mg and 27 mg, respectively. Before implantation surgery, all pellets were cleaned and chemically sterilized (Pellmar et al. 1999).

Pellet-implantation surgery. A rodent model system (AFRRI 1996), originally developed to mimic DU shrapnel loads seen in wounded personnel from the 1991 Persian Gulf War, was used to investigate the health effects of retained WA shrapnel. All rats were implanted with a total of 20 pellets split evenly between each hind leg. Experimental groups included Ta (negative control, 20 Ta pellets), low-dose WA (4 WA pellets and 16 Ta pellets), high-dose WA (20 WA pellets), and Ni (positive control, 20 Ni pellets). Tantalum was used as a negative implantation control because it is considered inert and has

been used in human prostheses (Hockley et al. 1990; Johansson et al. 1990). Nickel, a known carcinogen, was used as a positive control (Costa and Klein 1999; Kasprzak et al. 2003). Rats were implanted at 9 weeks of age. For the pellet implantation procedure, anesthesia was induced by continuous administration of isoflurane using an open circuit system with a scavenger/recapture system. All surgery was done using aseptic techniques. After the surgical sites were clipped and cleansed with Betadine, an incision was made through the skin to expose the gastrocnemius muscle. Pellets were implanted in the muscle, spaced approximately 1.5 mm apart on the lateral side of each leg. The incision was closed with sutures and tissue adhesive. Rats were closely monitored after surgery until they were ambulatory. An analgesic (buprenorphine hydrochloride; Reckitt and Colman, Hull, UK) was administered preoperatively and then as needed postoperatively. The surgical sites were examined daily for signs of inflammation, infection, and local metal toxicity.

Experimental groups. Our pellet implantation groups included Ta (negative control), WA (both a low- and high-dose group), and Ni (positive control). The original euthanasia time points were to be 1, 3, 6, 12, 18, and

24 months; however, because of the rapid tumor development, no WA- or Ni-implanted rat survived much past 6 months post-implantation. Final survival data therefore included rats originally assigned to the 12-, 18-, and 24-month experimental groups, whose animals died earlier than those designated time points. This resulted in group sizes of $n = 46$ for the Ta and both WA groups, and $n = 36$ for the Ni group. Hematologic assessments were conducted on the separate 1-, 3-, and 6-month WA implantation groups.

Pathology. At various times postimplantation or when moribund, rats were euthanized by isoflurane overdose. A complete gross pathology examination was conducted, noting any abnormalities, and tissues were collected for analysis. Weights of representative tissues, including spleen, thymus, testes, kidney, and liver, were determined and normalized to body weight. Tissues for histopathology were fixed in buffered formalin, processed and embedded in paraffin, cut at 5–6 μm , mounted, and stained with hematoxylin and eosin (H&E). Immunohistochemical analysis was conducted on 5- μm -thick sections of formalin-fixed, paraffinized tissue. After deparaffination and rehydration, nonspecific binding was blocked with Power Block (Biogenex, San

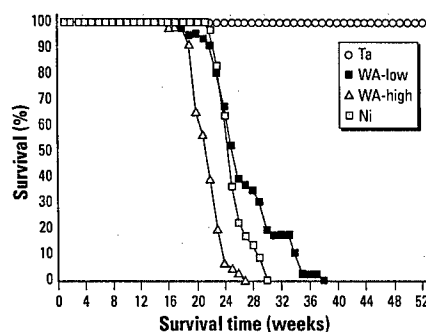


Figure 1. Survival times of pellet-implanted rats.

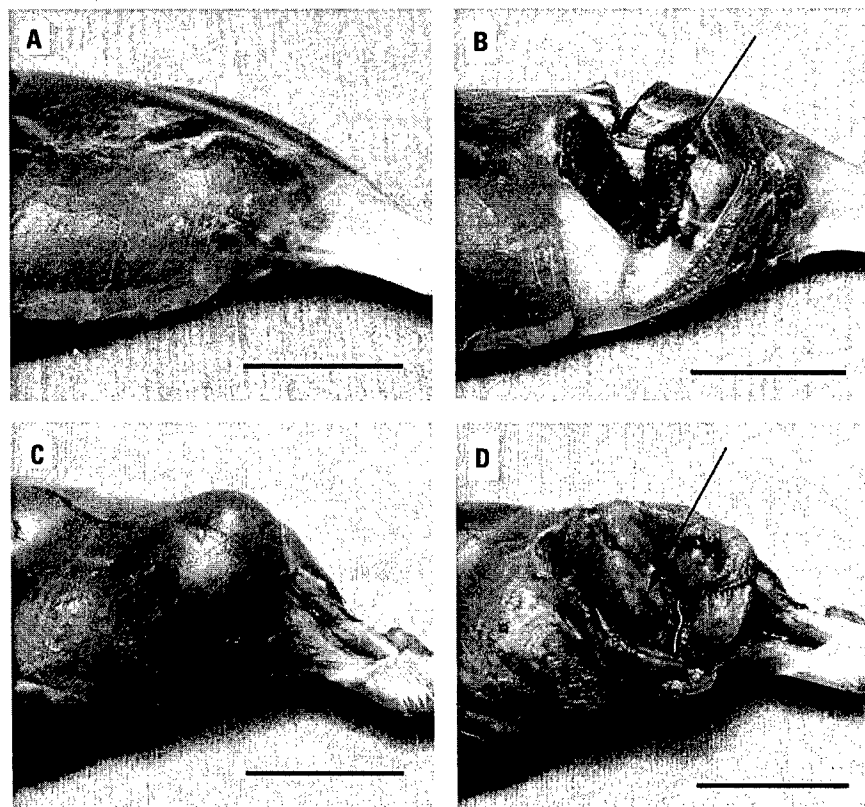


Figure 2. Effect of implanted WA pellets on F344 rats. (A) Gross appearance of Ta-implanted hind leg. (B) Dissected area around implanted Ta pellet (arrow indicates pellet). (C) Gross appearance of WA-implanted hind leg with tumor(s). (D) Dissected area around implanted WA pellet with tumor surrounding pellet (arrow indicates pellet). Bar = 2 cm.

Ramon, CA). The tissue was then reacted with prediluted rabbit anti-desmin polyclonal antibody (Biogenex) and treated with biotinylated secondary anti-rabbit antibody (Biogenex). After blocking with hydrogen peroxide, the tissue sections were labeled with peroxidase-conjugated streptavidin (Biogenex) and aminoethyl carbazole (AEC; Biogenex) was used as a chromogen. Slides were then counterstained with hematoxylin and mounted.

Hematology. At euthanasia, we obtained blood for hematologic assessments from the abdominal aorta of isoflurane-anesthetized rats using a heparinized needle and sample tubes containing EDTA (Becton-Dickinson, Franklin Lakes, NJ). We determined white and red blood cell counts; hemoglobin; hematocrit; mean corpuscular volume, hemoglobin, and hemoglobin concentration; red cell distribution width; platelet counts and volume; and neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts with a Bayer Advia 120 Hematology Analyzer (Bayer Diagnostics, Terrytown, NY).

Results

All rats tolerated the pellet implantation procedure with no apparent adverse effects. The incision sites were examined daily; no rat showed any signs of infection from the surgery, or any discomfort postoperatively. Body weights were recorded weekly. Once they had recovered from the surgical procedure, all rats gained weight at equivalent rates. However, in the first week after the pellet implantation surgery, the rate of weight gain by the Ta and low-dose WA rats was slower than normal, and high-dose WA and Ni rats lost weight. This was followed by large weight gains in postimplantation week 2 in all experimental groups. There were no statistical differences in rate of body weight gain between any of the groups throughout the remaining experimental period. As previously reported, the implantation and retention of cylindrical metal pellets (1 mm × 2 mm) had no effect on locomotive abilities in rats (AFRRI 1996; Pellmar et al. 1999), nor did we observe any such difficulties in this study.

At approximately 16–20 weeks post-implantation, we began to observe tumors at the pellet implantation sites in the WA and Ni rats. In some high-dose WA animals, palpable tumors were apparent as early as 14 weeks postimplantation. Tumors developed rapidly in WA-implanted animals. The tumors were aggressive and fast growing, necessitating euthanasia of the animals several weeks later. On the basis of previously published literature (Heath and Daniel 1964), we expected the Ni-implanted positive control rats to develop tumors at the implantation site, but the speed at which the tumors developed was surprising: approximately 5 months after implantation.

Figure 1 shows the percentage of surviving animals as a function of time after pellet implantation. Rats implanted with Ta pellets ($n = 46$) survived well beyond 12 months with no apparent health problems. All rats in the high- and low-WA and the Ni groups developed tumors and were euthanized upon becoming moribund. Rats in the high-dose WA group ($n = 46$) survived the least amount of time (mean survival time $\pm$ SD = 21.8 ± 2.1 weeks). Nickel-implanted animals ($n = 36$)

and the low-dose WA group ($n = 46$) survived slightly longer, with mean ($\pm$ SD) survival times of 25.4 ± 2.1 and 27.0 ± 4.6 weeks, respectively. The mean survival time of the high-dose WA animals was significantly shorter than that of the low-dose WA- or Ni-implanted animals [analysis of variance (ANOVA) followed by Dunnett's test, $p < 0.05$]. The mean survival times of the low-dose WA- and the Ni-implanted animals were not statistically different from each other. The

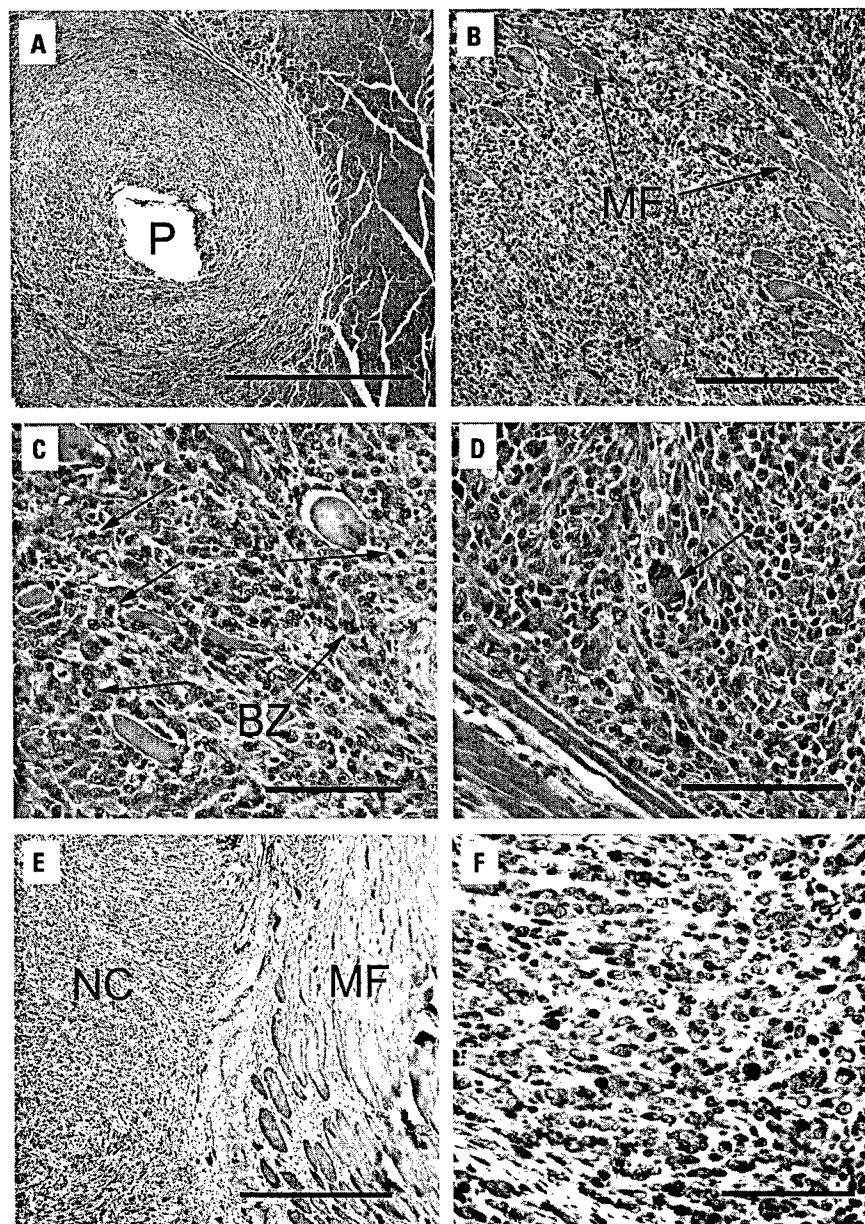


Figure 3. Histopathologic examination of leg tumor surrounding WA pellet. (A) H&E-stained section of leg tumor from F344 rat showing WA pellet hole (P); bar = 500 μ m. (B) H&E-stained tumor section showing neoplastic infiltration of preexisting muscle fibers (MF); bar = 200 μ m. (C) H&E-stained tumor section showing neoplastic cells with numerous mitoses (arrows) and bizarre mitotic figures (BZ); bar = 100 μ m. (D) H&E-stained tumor section showing pleomorphic cell (arrow); bar = 100 μ m. (E) Desmin staining of leg tumor showing neoplastic cells (NC) and muscle fibers (MF); bar = 500 μ m. (F) Desmin staining of neoplastic cells; bar = 50 μ m.

results reported here are part of a larger study that also investigated the health effect of embedded DU fragments. We did not observe tumor formation in the DU-implanted rats (Kalinich JF, Miller AC, McClain DE, unpublished data).

Upon euthanasia, the animals underwent necropsy, and tissue samples were taken for various analyses. Figure 2 shows the appearance of the hind limb of rats implanted with Ta (Figure 2A) or WA (Figure 2C) for 26 and 23 weeks, respectively, before surgical removal of the implanted pellets. The gross anatomy of the Ta-implanted leg is normal, whereas in the WA leg the tumor is clearly visible. Upon dissection, no obvious abnormalities were observed in the Ta-implanted animals, and the pellets could be easily removed (Figure 2B). However, in the WA-implanted animals, the pellets were surrounded by tumor (Figure 2D). In many cases, the interior of the tumor had become necrotic and/or hemorrhagic. Similar tumors were found for both WA- and Ni-implanted animals. In low-dose WA animals, tumors were found surrounding the WA pellets only. No tumors were found surrounding implanted Ta pellets. Implanted WA pellets rapidly oxidized and had a slightly eroded appearance. Ta pellets did not have an eroded appearance even after implantation for 6 months. However, despite their appearance, the WA pellets lost < 5% of their mass over this time.

Tumor tissue was histopathologically examined and characterized. Figure 3A shows the neoplastic cells surrounding the site of the implanted WA pellet. These cells infiltrated preexisting skeletal muscle fibers. Fibers that became isolated by this process degenerated and demonstrated a loss of cross-striations and internalization of nuclei (Figure 3B,C). Neoplastic cells were pleomorphic with marked anisocytosis and anisokaryosis (Figure 3D). In addition, an extremely high mitotic rate was observed in these cells, and bizarre mitoses were present. Immunohistochemical staining was used to determine the origin of these neoplastic cells. The cells were strongly positive for desmin (Figure 3E,F), suggesting a skeletal muscle origin.

In the WA-implanted animals, the tumors had metastasized to the lung. None of the Ni-implanted animals showed signs of lung metastases, although some exhibited endogenous histiocytic lipid pneumonia not seen in the WA animals. Figure 4A shows numerous metastatic foci in the lungs of a high-dose WA rat. These multiple masses obscure > 50% of the lung surface and up to 90% in the latter stages of development. Figure 4B shows a photomicrograph of these pulmonary metastases. Apparent is the multifocal, vascular orientation of these neoplasms. There are neoplastic cells surrounding the arterioles and

bronchioles, expanding the alveolar septae, and replacing alveolar spaces. These neoplastic cells have a high mitotic rate and are often seen surrounding or occluding arterioles (Figure 4C). Figure 4D shows that the metastatic neoplastic cells, as well as vascular and airway smooth muscle, are strongly positive for the muscle marker desmin.

Selected hematologic and organ weight parameters for euthanized rats are shown in Table 1. The Ta data were obtained from rats implanted with Ta pellets for 6 months. The data for the remaining groups were obtained at the time the rats became moribund because of tumor development. No significant differences in organ/body weight ratios were seen for the low-dose WA- or Ni-implanted animals compared with Ta-implanted control rats. However, high-dose WA-implanted rats showed significantly higher spleen:body weight ratios compared with control rats. In addition, thymus:body weight ratios were decreased in the high-dose WA rats. Because the spleen and thymus are integral components of the immune system, these changes suggest that embedded WA, at certain levels, may be immunotoxic. The kidney:body weight ratio for high-dose WA rats was also significantly higher than that

of Ta-implanted rats. High-dose WA rats euthanized 1 and 3 months after pellet implantation also exhibited significantly elevated spleen:body weight ratios compared with the appropriate Ta-implanted control rats (Tables 2 and 3). Thymus:body weight ratios, however, were not significantly different. At 3 months postimplantation, the kidney:body weight ratio in high-dose WA rats was significantly higher than that in Ta rats, but it was significantly lower at 1 month postimplantation. There were no 1- and 3-month Ni-implanted groups.

WA-implanted animals had significant changes in a number of hematologic parameters. Rats implanted with 20 WA pellets exhibited significant increases in white blood cell counts, red blood cell counts, hemoglobin, and hematocrit levels compared with Ta control rats, whereas rats implanted with 20 Ni pellets had significant decreases in red blood cell counts, hemoglobin, and hematocrit levels (Table 1). Hematologic parameters from low-dose WA rats were not statistically different from controls. Statistically significant increases in red blood counts, hemoglobin, and hematocrit levels were observed in high-dose WA animals as early as 1 month after pellet implantation and persisted throughout

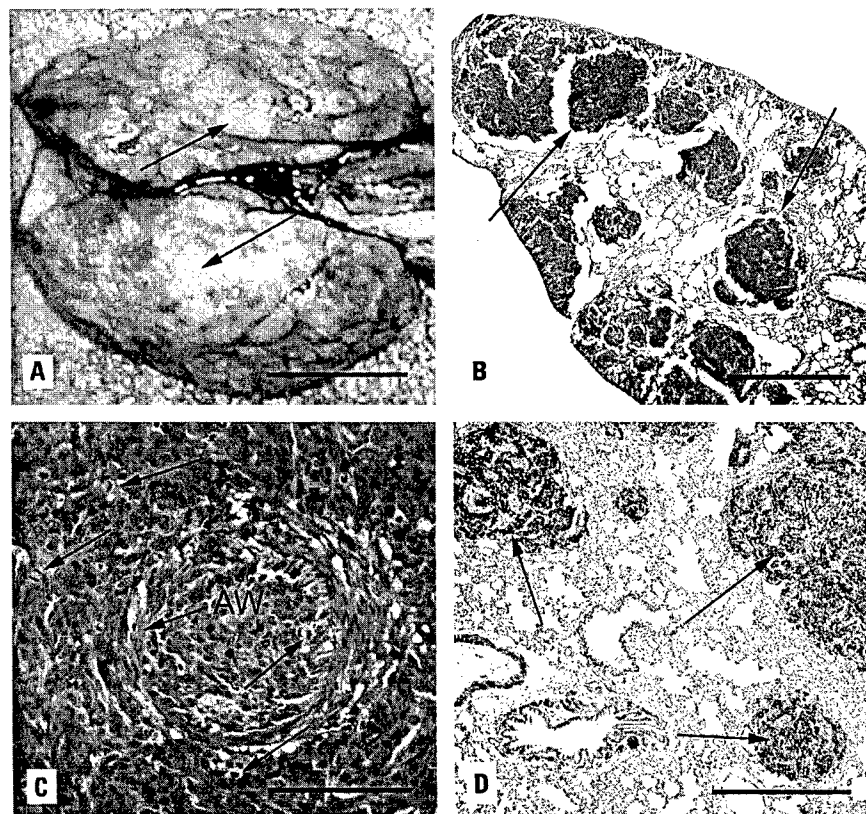


Figure 4. Lung metastases from WA-implanted F344 rats. (A) Gross appearance of pulmonary metastases from WA-implanted rat (arrows indicate metastatic foci); bar = 1 cm. (B) H&E-stained section of pulmonary metastases (arrows); bar = 1 mm. (C) H&E-stained section of an occluded pulmonary arteriole [arrow indicates vascular smooth muscle wall (AW)] showing neoplastic cells with numerous mitoses (arrows); bar = 50 μ m. (D) Desmin staining of pulmonary metastases (arrows); bar = 500 μ m.

the experimental period (Tables 2 and 3). In addition, there were statistically significant increases in the numbers of neutrophils, lymphocytes, monocytes, and eosinophils present in high-dose WA animals. Low-dose WA animals had elevated neutrophil, lymphocyte, and monocyte numbers at 3 months post-implantation, but only the neutrophil numbers were statistically different from the controls at the 5–6 month euthanasia point. The Ni-implanted animals had significantly lower lymphocyte counts than the controls. All other

parameters were statistically identical to the controls. These results suggest there is a dose-dependent perturbation in many hematology parameters as a result of an increasing WA pellet number.

Discussion

Tungsten-based alloys are currently being used as replacements for DU in kinetic-energy penetrators and for lead in small-caliber ammunition. However, the health effects of these unique alloys have not been investigated,

especially in the case of embedded fragments such as shrapnel wounds. In this study, using male F344 rats and a system designed to investigate the effects of embedded metal fragments (AFRRI 1996), we have shown the embedded weapons-grade WA (91.1% W, 6.0% Ni, 2.9% Co) results in rapid tumor formation at the implantation site in 100% of the rats. The rate of tumor formation correlates with pellet number. Ni-implanted rats also develop tumors at the implantation site, although not as rapidly as seen with WA. Histopathologic and immunohistochemical data support a diagnosis of a pleomorphic rhabdomyosarcoma for both the WA- and Ni-induced leg tumors (Altmannberger et al. 1985).

Rats implanted with 20 WA pellets (high-dose WA) showed significantly increased spleen:body weight ratios compared with Ta control rats. Low-dose WA rats (four WA pellets) also exhibited increased spleen:body weight ratios, but these increases were not statistically significant (ANOVA followed by Dunnett's test). Values for Ni-implanted rats were identical to control rats. The spleen changes observed in the high-dose WA rats were apparent as early as 1 month after pellet implantation. Once again, low-dose WA rats showed increased, but not statistically significant, spleen:body weight ratios. With the exception of the spleen, the only other organ:body weight perturbations were seen in high-dose WA rats and included a decrease in thymus:body weight ratio at approximately 5 months and changes in kidney:body weight ratios. The 1-month kidney:body weight ratio for high-dose WA rats was significantly lower

Table 1. Selected hematologic and organ weight parameters (mean $\pm$ SEM) for euthanized rats.

	Ta	WA (low)	WA (high)	Ni
White blood cells ($10^3/\mu\text{L}$)	3.19 $\pm$ 0.24	3.95 $\pm$ 0.43	4.56 $\pm$ 0.29*	2.56 $\pm$ 0.20
Red blood cells ($10^6/\mu\text{L}$)	8.32 $\pm$ 0.09	8.03 $\pm$ 0.19	10.10 $\pm$ 0.07**	7.46 $\pm$ 0.13**
Hemoglobin (g/dL)	14.50 $\pm$ 0.13	13.90 $\pm$ 0.36	16.46 $\pm$ 0.30**	12.95 $\pm$ 0.23**
Hematocrit (%)	41.77 $\pm$ 0.53	40.38 $\pm$ 0.96	50.18 $\pm$ 0.39**	38.12 $\pm$ 0.77**
MCV (fL)	50.22 $\pm$ 0.16	50.26 $\pm$ 0.28	49.71 $\pm$ 0.16	51.08 $\pm$ 0.66
MCH (pg)	17.46 $\pm$ 0.15	17.31 $\pm$ 0.13	16.30 $\pm$ 0.28**	17.35 $\pm$ 0.08
MCHC (g/dL)	34.77 $\pm$ 0.36	34.46 $\pm$ 0.32	32.81 $\pm$ 0.62**	34.05 $\pm$ 0.50
RDW (%)	12.54 $\pm$ 0.09	13.07 $\pm$ 0.11**	13.77 $\pm$ 0.09**	13.04 $\pm$ 0.16*
Platelets ($10^3/\mu\text{L}$)	562.00 $\pm$ 14.72	542.05 $\pm$ 14.27	467.50 $\pm$ 17.57**	487.18 $\pm$ 26.10*
MPV (fL)	9.93 $\pm$ 0.69	8.64 $\pm$ 0.52	10.13 $\pm$ 0.62	8.97 $\pm$ 0.52
Neutrophils ($10^3/\mu\text{L}$)	0.79 $\pm$ 0.05	1.03 $\pm$ 0.09*	1.31 $\pm$ 0.12**	0.78 $\pm$ 0.09
Lymphocytes ($10^3/\mu\text{L}$)	2.21 $\pm$ 0.18	2.42 $\pm$ 0.17	2.95 $\pm$ 0.23*	1.63 $\pm$ 0.12*
Monocytes ($10^3/\mu\text{L}$)	0.07 $\pm$ 0.01	0.09 $\pm$ 0.02	0.13 $\pm$ 0.02*	0.05 $\pm$ 0.01
Eosinophils ($10^3/\mu\text{L}$)	0.08 $\pm$ 0.01	0.08 $\pm$ 0.01	0.12 $\pm$ 0.01**	0.06 $\pm$ 0.01
Basophils ($10^3/\mu\text{L}$)	0.02 $\pm$ 0.00	0.03 $\pm$ 0.00	0.03 $\pm$ 0.00	0.02 $\pm$ 0.00
Spleen (mg/g bw)	2.18 $\pm$ 0.10	2.30 $\pm$ 0.08	2.60 $\pm$ 0.06**	2.17 $\pm$ 0.05
Thymus (mg/g bw)	0.86 $\pm$ 0.03	0.76 $\pm$ 0.04	0.70 $\pm$ 0.04*	0.74 $\pm$ 0.07
Liver (mg/g bw)	29.21 $\pm$ 0.28	29.39 $\pm$ 0.24	28.77 $\pm$ 0.35	29.52 $\pm$ 0.39
Kidney (mg/g bw)	5.13 $\pm$ 0.06	5.13 $\pm$ 0.06	5.36 $\pm$ 0.05*	5.15 $\pm$ 0.08
Testes (mg/g bw)	7.31 $\pm$ 0.07	7.20 $\pm$ 0.08	7.40 $\pm$ 0.10	7.21 $\pm$ 0.14

Abbreviations: bw, body weight; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RDW, red blood cell distribution width. Data represent mean $\pm$ SEM of 20 observations (10 for Ni group).

* $p < 0.05$, and ** $p < 0.01$ compared with the Ta control group by one-way ANOVA followed by Dunnett's test for group mean comparisons.

Table 2. Selected hematologic and organ weight parameters (mean $\pm$ SEM) for rats implanted with metal pellets for 3 months.

	Ta	WA (low)	WA (high)
White blood cells ($10^3/\mu\text{L}$)	2.88 $\pm$ 0.20	4.06 $\pm$ 0.14**	4.01 $\pm$ 0.21**
Red blood cells ($10^6/\mu\text{L}$)	7.48 $\pm$ 0.06	8.48 $\pm$ 0.15*	9.10 $\pm$ 0.70**
Hemoglobin (g/dL)	12.90 $\pm$ 0.09	15.48 $\pm$ 0.35*	17.29 $\pm$ 0.15**
Hematocrit (%)	38.10 $\pm$ 0.27	42.14 $\pm$ 0.73*	44.79 $\pm$ 0.62**
MCV (fL)	50.96 $\pm$ 0.45	49.70 $\pm$ 0.09	48.87 $\pm$ 0.39
MCH (pg)	17.26 $\pm$ 0.12	18.27 $\pm$ 0.17	17.65 $\pm$ 0.12
MCHC (g/dL)	33.84 $\pm$ 0.35	36.71 $\pm$ 0.31**	35.89 $\pm$ 0.31**
RDW (%)	12.82 $\pm$ 0.33	12.68 $\pm$ 0.12	13.61 $\pm$ 0.09**
Platelets ($10^3/\mu\text{L}$)	513.20 $\pm$ 38.36	585.11 $\pm$ 35.87	568.29 $\pm$ 8.82
MPV (fL)	9.58 $\pm$ 1.13	9.14 $\pm$ 0.59	11.74 $\pm$ 0.51
Neutrophils ($10^3/\mu\text{L}$)	0.62 $\pm$ 0.04	0.79 $\pm$ 0.03*	0.91 $\pm$ 0.08*
Lymphocytes ($10^3/\mu\text{L}$)	2.10 $\pm$ 0.16	3.06 $\pm$ 0.14*	2.82 $\pm$ 0.17*
Monocytes ($10^3/\mu\text{L}$)	0.04 $\pm$ 0.01	0.07 $\pm$ 0.01*	0.08 $\pm$ 0.01*
Eosinophils ($10^3/\mu\text{L}$)	0.09 $\pm$ 0.01	0.09 $\pm$ 0.01	0.09 $\pm$ 0.01
Basophils ($10^3/\mu\text{L}$)	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.02 $\pm$ 0.00
Spleen (mg/g bw)	2.07 $\pm$ 0.03	2.16 $\pm$ 0.03	2.50 $\pm$ 0.03**
Thymus (mg/g bw)	0.73 $\pm$ 0.03	0.84 $\pm$ 0.03	0.70 $\pm$ 0.04
Liver (mg/g bw)	30.58 $\pm$ 0.33	31.00 $\pm$ 0.33	30.27 $\pm$ 0.31
Kidney (mg/g bw)	5.43 $\pm$ 0.06	5.73 $\pm$ 0.23	5.76 $\pm$ 0.04**
Testes (mg/g bw)	8.34 $\pm$ 0.12	8.21 $\pm$ 0.46	8.42 $\pm$ 0.18

Abbreviations: bw, body weight; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RDW, red blood cell distribution width. Data represent mean $\pm$ SEM of 15 observations.

* $p < 0.05$, and ** $p < 0.01$ compared with the age-matched Ta control group by one-way ANOVA followed by Dunnett's test for group mean comparisons.

Table 3. Selected hematologic and organ weight parameters (mean $\pm$ SEM) for rats implanted with metal pellets for 1 month.

	Ta	WA (low)	WA (high)
White blood cells ($10^3/\mu\text{L}$)	3.86 $\pm$ 0.20	3.81 $\pm$ 0.14	3.86 $\pm$ 0.21
Red blood cells ($10^6/\mu\text{L}$)	7.84 $\pm$ 0.08	7.74 $\pm$ 0.07	8.50 $\pm$ 0.07**
Hemoglobin (g/dL)	13.65 $\pm$ 0.15	14.81 $\pm$ 0.16	15.84 $\pm$ 0.14**
Hematocrit (%)	40.15 $\pm$ 0.42	39.66 $\pm$ 0.50	43.29 $\pm$ 0.35**
MCV (fL)	51.20 $\pm$ 0.14	51.22 $\pm$ 0.31	50.98 $\pm$ 0.19
MCH (pg)	17.41 $\pm$ 0.05	19.12 $\pm$ 0.09	18.64 $\pm$ 0.19**
MCHC (g/dL)	34.01 $\pm$ 0.12	37.37 $\pm$ 0.29	36.56 $\pm$ 0.41**
RDW (%)	12.21 $\pm$ 0.11	12.69 $\pm$ 0.11	14.18 $\pm$ 0.18**
Platelets ($10^3/\mu\text{L}$)	646.50 $\pm$ 18.76	641.00 $\pm$ 17.97	756.20 $\pm$ 43.48*
MPV (fL)	7.91 $\pm$ 0.40	8.56 $\pm$ 0.39	9.90 $\pm$ 0.55*
Neutrophils ($10^3/\mu\text{L}$)	0.65 $\pm$ 0.04	0.79 $\pm$ 0.05	0.81 $\pm$ 0.04**
Lymphocytes ($10^3/\mu\text{L}$)	3.04 $\pm$ 0.18	2.85 $\pm$ 0.13	2.90 $\pm$ 0.18
Monocytes ($10^3/\mu\text{L}$)	0.06 $\pm$ 0.00	0.06 $\pm$ 0.01	0.07 $\pm$ 0.00
Eosinophils ($10^3/\mu\text{L}$)	0.07 $\pm$ 0.01	0.08 $\pm$ 0.01	0.05 $\pm$ 0.00*
Basophils ($10^3/\mu\text{L}$)	0.02 $\pm$ 0.00	0.02 $\pm$ 0.00	0.01 $\pm$ 0.00
Spleen (mg/g bw)	2.37 $\pm$ 0.06	2.42 $\pm$ 0.05	2.73 $\pm$ 0.04**
Thymus (mg/g bw)	1.07 $\pm$ 0.03	1.14 $\pm$ 0.04	1.06 $\pm$ 0.03
Liver (mg/g bw)	34.47 $\pm$ 0.26	34.31 $\pm$ 0.22	34.18 $\pm$ 0.61
Kidney (mg/g bw)	6.17 $\pm$ 0.08	6.06 $\pm$ 0.06	5.91 $\pm$ 0.05*
Testes (mg/g bw)	10.10 $\pm$ 0.16	9.86 $\pm$ 0.13	9.98 $\pm$ 0.11

Abbreviations: bw, body weight; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RDW, red blood cell distribution width. Data represent mean $\pm$ SEM of 15 observations.

* $p < 0.05$, and ** $p < 0.01$ compared with the age-matched Ta control group by one-way ANOVA followed by Dunnett's test for group mean comparisons.

than control. However, from 3 months on, these ratios were significantly higher than control. It is possible that the lower kidney weights at 1 month postimplantation represent a toxic response to the heavy metals from the implanted pellets, but by 3 months and later, the kidney has begun to respond in a different manner. Although there were no gross abnormalities of the kidney at necropsy, we continue to investigate this observation.

A variety of hematologic changes were observed in WA- and Ni-implanted rats. Ni-implanted rats showed a significant decrease in red blood cells, hemoglobin, and hematocrit at the time of morbidity, indicating possible Ni-induced anemia. For low-dose WA rats the hematologic changes, including significant increases in red blood cells, white blood cells, hemoglobin, hematocrit, neutrophils, lymphocytes, and monocytes, peaked at 3 months postimplantation and returned to normal by 5–6 months. High-dose WA rats demonstrated the same changes observed in low-dose WA rats, but they occurred much more rapidly (as early as 1 month postimplantation) and persisted throughout the life of the animal. The splenomegaly and hematologic changes observed in these rats are suggestive of polycythemia. Cobalt has been used experimentally to induce polycythemia in rats (Endoh et al. 2000; Rakusan et al. 2001), although the concentration required is far greater than found in the WA pellets. In addition, the speed at which these hematologic changes occurred in the high-dose WA rats was also surprising. These results suggest a dose-dependent perturbation in many hematology parameters as a result of an increasing WA pellet number.

The search for munitions that are considered environmentally friendly yet still retain their military effectiveness has led to the appearance of many unique alloys on the modern battlefield. Often, decisions on the health consequences of exposure (inhalation, ingestion, wound contamination, etc.) to these specific alloys are based on studies that investigated only one specific metal of the alloy rather than the particular alloy in question. Tungsten-based munitions are a recent addition to many countries' arsenals, primarily in response to the continuing concerns regarding the potential environmental and health effects of DU in kinetic-energy penetrators and of lead in small-caliber ammunition. For years, exposure to tungsten was thought to be of little consequence to health. In fact, tungsten is occasionally found as a minor component in some of the various alloys used to produce medical implant devices such as artificial hips and knees. The tungsten concentration in these alloys ranges from 5% to 15%. Because the alloy used in WA munitions usually contains > 90% tungsten, along with smaller amounts of other metals, it was also assumed that exposure to these

alloys would present little or no health risk. As we have shown here, this is not the case in our rodent model. Embedded WA pellets not only resulted in aggressive, metastatic, pleomorphic rhabdomyosarcomas, but also caused significant hematopoietic changes well before the carcinogenic effect was observed. It seems unlikely that these adverse health effects can be attributed solely to the small amounts of Ni and/or Co present in the alloy. The tumors induced by the 100% Ni implants occurred later than those induced by the alloys containing 6% Ni. However, recent *in vitro* studies have demonstrated a synergistic effect in terms of damage when tungsten is present with these metals (Miller et al. 2001, 2002).

The mechanism of the effects reported here with embedded WA pellets remains unclear. Despite the fact that the smooth and impermeable surface of the pellets represent characteristics known capable of inducing foreign-body or solid-state carcinogenesis (Bates and Klein 1966; Brand et al. 1975), this process is unlikely to have occurred in our experiments because implanted Ta pellets of an identical geometry and surface resulted in no tumor formation. One possibility is that free-radical reactions at the interface of the pellet and tissue could result in damage leading to carcinogenesis. Recently, the role of tungsten in human health and disease has come under increased scrutiny. Environmental testing of the leukemia cluster around Fallon, Nevada, in the United States showed slightly elevated levels of several heavy metals including uranium and Co but significantly elevated levels of tungsten [Centers for Disease Control and Prevention (CDC) 2003]. Although no definitive link between elevated tungsten levels and cancer has been established, because of the uncertainty surrounding this issue, the U.S. National Toxicology Program recently added tungsten to their list of compounds to be assessed for adverse health effects. Further study of the health effect of tungsten and WAs is clearly indicated.

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