UNITED STATES OF AMERICA
NUCLEAR REGULATORY COMMISSION

BEFORE THE ATOMIC SAFETY AND LICENSING BOARD

In the Matter of	)	
	)	
CROW BUTTE RESOURCES, Inc.	)	Docket No. 40-8943
	)	
	)	ASLBP No. 08-867-OLA-BD01
(License Renewal for the In Situ	)	
Leach Facility, Crawford, Nebraska	)	September 3, 2008

Attached are copies of the exhibits that were originally submitted with the Tribe's Petition to Intervene that apparently did not reach all parties. The Tribe apologizes for any inconvenience or confusion.

Respectfully submitted this 3rd day of September, 2008.

/s/ Elizabeth Maria Lorina
/s/ Mario Gonzalez
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CERTIFICATE OF SERVICE

I hereby certify that copies of the forgoing RESEND OF EXHIBITS have been served upon the following persons by Electronic Information Exchange.

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Copy: Bill (Rudy) 24 April 89 recvd 24 A
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4 April 1989

RECEIVED RECEIVED
APR 27 1989 APR 29 1989
DEPARTMENT OF ENVIRONMENTAL CONTROL DEPARTMENT OF ENVIRONMENTAL CONTROL

Mr. Gary Konwinski
Nuclear Regulatory Commission
Uranium Recovery Field Office
P. O. Box 25325
Denver, CO 80225

Dear Mr. Konwinski:

I am writing to you to express my concern regarding the probability of ground water contamination in the course of on-going and anticipated in situ uranium mining operations in Dawes County, Nebraska. These operations are directed by Ferret Exploration Company of Nebraska with joint venture support from Uranerz USA, Inc. 165 S Union Blvd., Lakewood, CO.

I am personally acquainted with the circumstances which are described herein through my former affiliation with Uranerz. By way of establishing my credentials, I have been an exploration geologist for nearly twenty years. I have been invloved in uranium exploration for the past fourteen years. During my employment by Uranerz I had the opportunity to examine the exploration data of the Crow Butte area in the course of my normal duties, and, in fact, my opinion concerning the interpretation of the Crow Butte data was specifically sought by Uranerz management within the last year. I wish to emphasize that this letter is certainly not resultant from anti-mining or anti-uranium sentiment. I am both in favor of a strong mining industry and a healthy nuclear power industry. Rather, I believe that certain aspects of the geology of the Crow Butte uranium deposits have been deliberately overlooked or surpressed so that mining could proceed and profits be gained regardless of the effect upon local ground water quality. In my opinion, such actions ultimately work to the detriment of those of us in mining who make good faith efforts to maintain environmental quality.

As you are aware, geologic interpretation is rarely based upon direct observation of all the necessary data, but rather relies heavily upon indirect evidence and inductive reasoning. Certainly, it can be difficult to arrive at final answers under such conditions. The amount of information that is now available in the general Crow Butte area is great enough to minimize the uncertainty of geologic interpretation to the point that certain probabilities (not possiblities) may be stated.

It is my understanding that geologists of the Nebraska State agencies involved in permitting believed that structural control of the Crow Butte mineralization was likely, but were ultimately dissuaded from that belief by Ferret personnel. In fact, it is my understanding that mining was only allowed to proceed because structural control was finally ruled out. I have no way of knowing exactly what information was used to arrive at that evaluation, but I can state that as a matter of my professional opinion I find it to be highly probable that most, if

not all, uranium mineralization in the Crow Butte area is directly and primarily controlled by near-vertical faults cutting through the area.

The evidence for such faulting may be found only by detailed evaluation of drilling results, and may be summarized as follows:

1. Uranium mineralization occurs in well-defined, NW-SE linear zones (an alternate interpretation relating linear trends to a redox boundary is unlikely since oxidized facies are not present and since the ore zones exhibit such a high degree of "straightness" that a lithologic or chemical boundary could not be the cause).

2. Structure profiles drawn at right angles to mineral trends show abrupt vertical offset of marker beds in a fashion that can only be explained by repetitive faulting after deposition of the marker beds. This faulting corresponds in location to the zones of strongest mineralization.

3. Mineral trends are coincident with and parallel to surficial geomorphic features which are most likely due to fault control of erosional patterns.

4. More subjective interpretaions using isopacheous and paleomorphologic interpretaions are consistent with faulting during (?) and after Basal Chadron deposition.

Mr. Stephen P. Collings of Ferret and Mr. Karl Kegel, President of Uranerz USA, Inc. were made aware of the liklihood of structural control by means of technical memoranda written in July 1988 by another geologist in the Uranerz organization. This person would have reason to fear retribution if he made his own views known to regulatory agencies. Since I am separated from Uranerz however, I am free to act. Mr. Kegel and Mr. Collings along with Mr. H. Akin, who is the Uranerz Vice President in charge of mining operations, and who has immediate supervisory responsibility on behalf of Uranerz have apparently agreed to surpress general knowledge of the structural interpretation so that mining and exploration may proceed unimpeded.

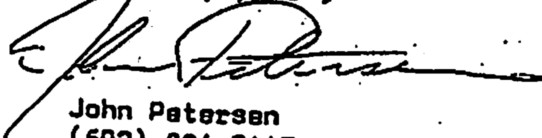
It is true that hardly an area exists that is not somehow affected by faulting. For example, the Uranerz North Butte property in Campbell County, Wyoming is also a potential <u>in situ</u> property. There, certain drill hole data have been surpressed in the preparation of a similarity document (c.f. Ruth) because they indicate, but do not prove, that faults may simply occur. In contrast, the Crow Butte area faults not only exist, but they <u>control</u> mineralization. The significance is obvious. Near-vertical, secondary porosity that is provided by such faults make for natural and effective zones for ground water movement and also for the movement of uranium-laden solvents injected into the ore zone in the course of mining. Under these circumstances, the contamination of suprajacent, and to some extent, subjacent, aquifers becomes possible, if not likely.

It is my understanding that Ferret, with the approval of Uranerz top management, has refused to undertake specifically designed drilling to investigate the significance of the structural control of mineralization. Clearly, Ferret and Uranerz will choose to ignore the existance of faults and their significance in relation to ground water quality unless they are forced to address the issue either by enforcement of regulation, or perhaps, if that is not forthcoming, by public pressure.

I believe that the Nebraska Department of Environmental Control and the Nuclear Regulatory Commission should require specific investigations to evaluate the significance of faulting in relation to ground water quality and that mining should be suspended until it can be shown that uranium mining has not and will not cause ground water contamination.

Unfortunately, I can not provide you with the actual exploration data, being proprietary. If you wish to discuss this letter or my conclusions you may reach me at the number below. I feel that I am ethically bound to report my professional assessment in this matter to you, I hope that it is sufficient to encourage you to seek the kind of detailed information you will need to make your own assessment.

Sincerely yours,



John Petersen
(602) 824-3447

XC: Nebraska Department of Environmental Control.

Geochemical Consulting Services, LLC
Solubility, Speciation, and Reaction-Path Modeling
Groundwater and Soil Geochemistry
Environmental Assessment
Risk Assessment

July 28, 2008

David Cory Frankel
Counsel for Sioux Nation

Mr. Frankel:

I have reviewed documents associated with the Renewal of the Source Material License (No. SUA-1534) for Crow Butte, Nebraska and have found the applicant has not provided sufficient data to demonstrate proper development of (1) baseline water quality in the aquifer exemption zone and (2) excursion limits at monitoring wells.

Baseline Water Quality in the Aquifer Exemption Zone

Figure 2.9-2 illustrates the proposed aquifer exemption zone around the ore bodies, and there is no statistical justification for the location of the baseline wells to validate that the results in Table 2.9-4 represent the water quality in the exempt zone. Note that the baseline wells in Figure 2.9-2 are clustered and not spread out over the entire exempt zone, and this violates statistical protocol. Within the aquifer exemption zone (i.e., the zone within the monitoring well ring), a systematic grid must be laid out to determine the location of the baseline water-quality wells. The density of nodes within the grid will be determined by the size of the area and the data quality objectives. Data quality objectives (EPA 2000a & 2000b) state the statistical confidence one wishes to have in the estimate of the mean (normal or log normal distribution) or median (no defined distribution) for the water-quality parameters. If a high level of confidence is required for an estimate of the mean or median, more baseline wells will be required.

For example, if we wish to establish a 95 percent confidence interval on the mean for a normal or log normal set of data, with an estimated standard deviation of 20 and using a half width of 10 for the confidence interval, an exempt aquifer area measuring 1200 by 1200 feet would require a minimum of 9 baseline wells (PNNL, 2007). The location of the wells on the grid nodes is illustrated on Figure 1. The half width of the confidence level is a key consideration in determining the number of wells; as the half width decreases, the number of wells increases. The selected level of confidence and estimated standard deviation also affect the number of wells. A lower level of confidence and lower estimate of the standard deviation would result in fewer baseline wells. Alternatively, we may elect to locate wells on the grid using a random number generator. In general, less wells are required if they are located randomly, and this is shown on

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Figure 1 as two random locations per quadrant. Free modeling software, developed by the Pacific Northwest National Laboratory for the Department of Energy (PNNL 2007), allows a large number of scenarios to be evaluated to determine the optimum data quality objectives and sampling approach for the stakeholders.

The well logs provided indicate the Chadron is approximately 50 to 80 feet thick through most of the mining area (Figures 2.6-4 through 2,6-11). The sampling interval for the baseline wells is 20 feet (Table 2.9-3), which does not represent the entire thickness of the aquifer. Figure 2 shows that a water sample obtained from Well 1, screened only in the ore zone, returns a biased sample that does not represent the water quality of the column of water at the given location. Well 2 (Figure 2) indicates the correct method for sampling the water, which requires that the entire thickness of the aquifer be screened to obtain a representative sample.

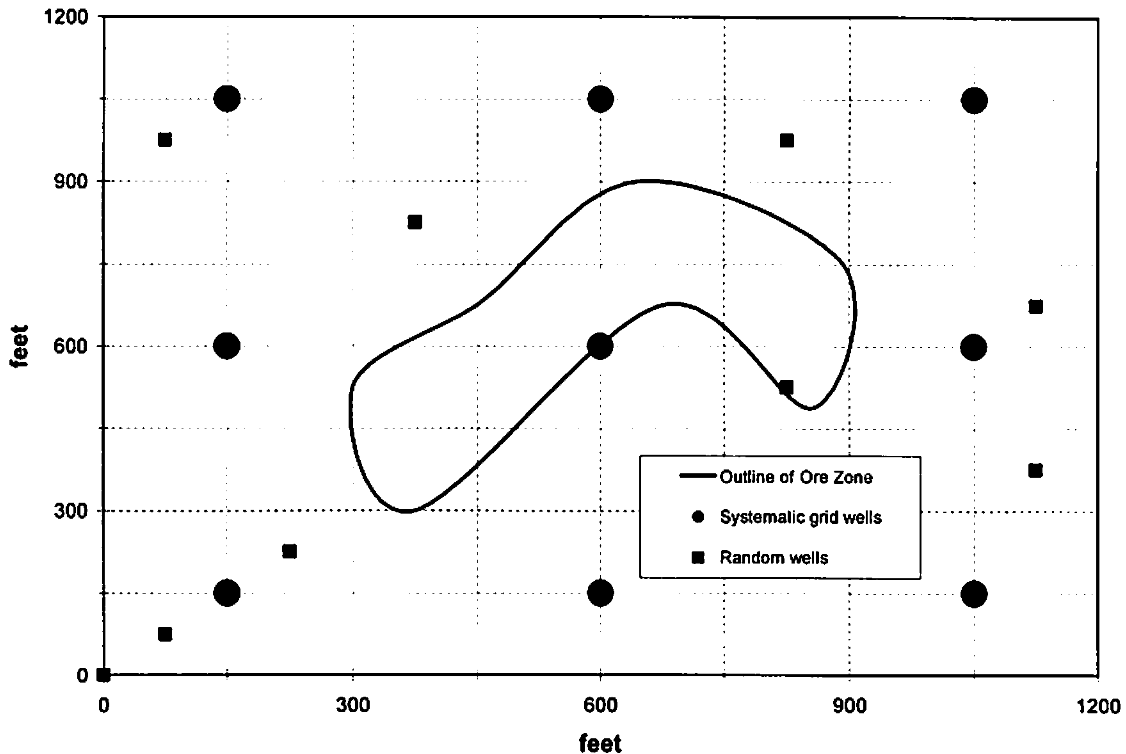

Figure 1. Chart of aquifer exempt zone (i.e., zone surrounded by monitoring wells) and locations for baseline wells established with valid statistical methods.

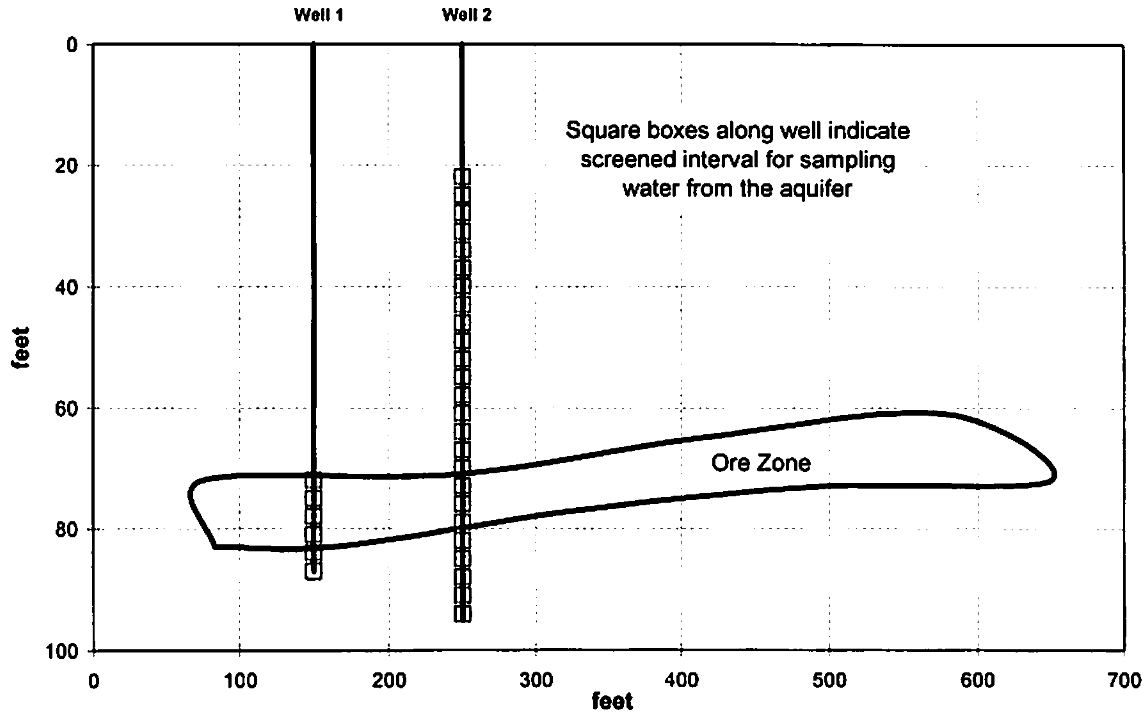

Figure 2. Cross section of an aquifer (20 to 90 feet) showing improper (Well 1) and proper (Well 2) sampling intervals to obtain a representative sample.

The number of sampling events used to establish the aquifer water quality in Table 2.9-4 and the analytical results for each sampling event are not provided to evaluate the results in the table. A minimum of 4 sampling rounds should be collected, and EPA recommends 8 rounds with sampling occurring no more frequently than once monthly.

After collecting a round of data, a proper statistical analysis must be performed to obtain a valid estimate of the mean or median for the water-quality parameter. The first statistical test that must be performed is to evaluate whether the data follow a normal or log normal distribution, and this can be done with the Shapiro-Wilk test or a probability plot (EPA 1992). If the data fail to follow a normal or log normal distribution, non-parametric methods must be used to estimate the median and confidence intervals. The importance of establishing the data distribution is summarized in Table 1.

Assume nine samples were taken from the nine locations on Figure 1 and analyzed for radium-226. A valid statistical sampling of the exempt aquifer zone collects more

samples from outside the ore zone than within the ore zone, as the greater area within the monitoring well ring is outside the ore zone. Reported results, mean and median values, and probability scores from the Shapiro-Wilk test are given in Table 1. Note the significant difference between the mean (exceeds EPA drinking water standard) and median (below EPA drinking water standard) values, and this is a fairly good indication that the data do not follow a normal or log normal distribution.

A probability plot of the data (Figure 3) and the results of the Shapiro-Wilk test confirm this. The normal-quantile values must fall on a straight line or the Shapiro-Wilk probability values must exceed 0.05 (at the 95 percent confidence level) for a normal or log normal distribution to be declared, which is clearly not the case. Therefore, the median, and not the mean, must be used to represent the central tendency of the data. Note that inappropriate use of the mean results in a high bias on the estimate of the baseline value for radium-226, which improperly elevates restoration clean-up levels and lowers the costs associated with the number of pore volumes needed to exchange to meet the clean-up levels.

Table 1. Radium-226 values and statistical results.

Radium-226 (pCi/L)	Mean (pCi/L)	Median (pCi/L)	Shapiro-Wilk test
0.8			Probability result
0.9	12	2.3	
1.1			Normal
1.7			$P < 0.01$
2.3			
2.8			Log normal
3.1			$P = 0.02$
5.2			
87			

The arguments presented above for Section 2.9-3 of the License Renewal Application also hold for the baseline and restoration values presented for the mining units (Tables 2,7-6 through 2.7-15 and Tables 6.1-2 through 6.1-11). That is, all data and methods used to construct baseline and restoration values must be included in the application to allow an independent evaluation of the summary tables and valid statistical protocols must be used to locate the wells and evaluate the analytical results. Baseline and restoration values presented in the application are improperly biased to high results, and this allows restoration to be achieved with less cost and time at the expense of greater contamination in the aquifer.

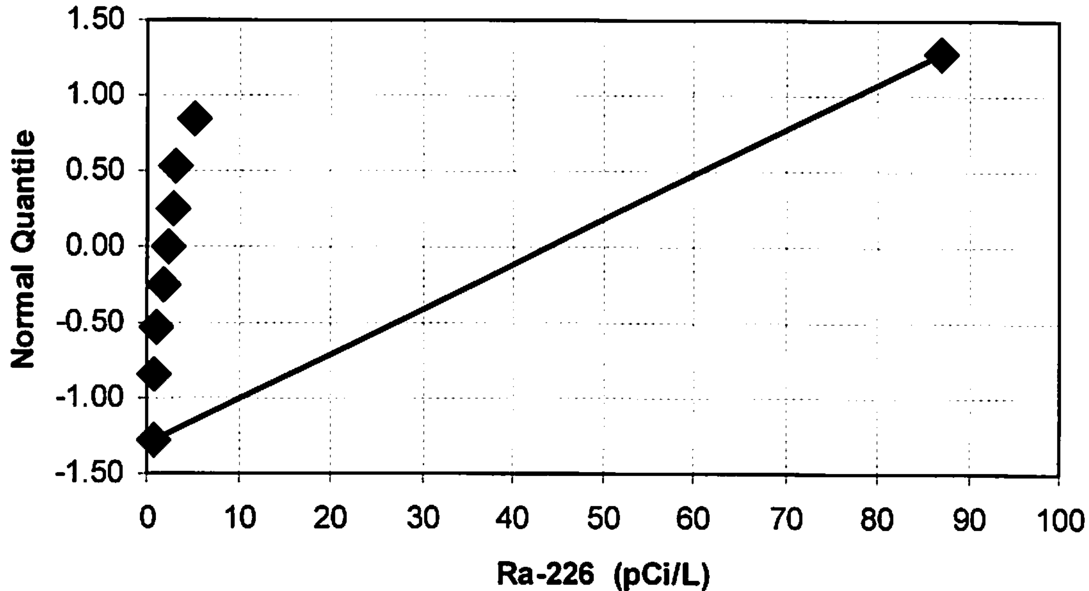

Figure 3. Probability plot indicating that all data do not lie near or on a straight line, which rules out a normal or log normal distribution.

Excursion Limits at Monitoring Wells

Section 5.8.8.2 briefly touches on baseline water quality for the monitoring wells, and upper control limits for indicating an excursion. Baseline water quality is determined on three samples collected 14 days apart, and this is inconsistent with the best practice and guidance discussed above. Chloride, conductivity and alkalinity are noted as the parameters used to monitor lixiviant migration. As uranium is mobilized and transported by the high oxygen and alkalinity in the lixiviant, there is no valid scientific reason to exclude it from the list of excursion monitoring parameters. Upper control limits are set at 20 percent above the maximum baseline value for parameters that exceed 50 mg/L, and for parameters below 50 mg/L 5 standard deviations or 15 mg/L is added to the average value for the indicator. There is no discussion of a valid statistical approach to justify the method for calculating upper control limits.

Ground-water quality data from the monitoring wells must be evaluated to determine if a normal or log normal distribution is present (see discussion above). If the data fail to

follow a normal or log normal distribution, the mean and standard deviation cannot be used and non-parametric methods must be employed to develop the upper control limit for the excursion parameters.

Uranium is a key indicator of lixiviant excursion because its concentration in baseline wells is generally two or three orders of magnitude lower than the lixiviant and it is highly mobile as a carbonate complex in the lixiviant. Comparing Table 2.7-15 with Table 3.1-3 shows that the lixiviant/baseline concentration ratio is 27 for chloride, 11 for conductivity, 13 for alkalinity and 1300 for uranium (the higher the lixiviant/baseline ratio, the greater the probability that an excursion will be detected at a monitoring well). As the uranium ratio is approximately 100 times greater than the other parameters, it will perform about 100 times better in the detection of an excursion. Therefore, there is no rationale basis to exclude the best excursion indicator from the list of excursion parameters.

EPA (1992) discusses the proper statistical calculation of tolerance limits (a.k.a. upper control limits) using parametric (normal or log normal) and non-parametric techniques. In general, 3 or 4 samples are not sufficient to establish a normal or log normal distribution, and EPA recommends that a non-parametric tolerance limit be set at the maximum observed value (not the maximum value plus 20 percent). As more data are collected at the monitoring well, the distribution of the data is rechecked and if a normal or lognormal distribution is indicated, a tolerance limit can be calculated using the equations provided by EPA (1992). There is no basis or justification for calculating an upper control limit by adding 15 mg/L to the average value. Additionally, using 5 standard deviations added to the average applies only if the data follow a normal or log normal distribution and a Shewhart control chart is constructed.

EPA (1992) addresses the use of 4.5 standard deviations added to the mean via the construction of a Shewhart-cumulative sum control chart. The use of this approach is recommended provided that the data follow a normal or log normal distribution. Assuming a sufficient number of samples have been collected at a monitoring well to demonstrate that the measured values follow a normal distribution, two statistical parameters are calculated to evaluate contaminate migration at the well. First, the standardized mean is calculated from the mean and standard deviation (EPA 1992) and compared to the Shewhart control limit (SCL; set at 4.5 standard deviations above the mean) to evaluate a rapid increase in concentration at the monitor well. Second, the cumulative sum (CUSUM; set at 5 standard deviations above the mean) of the standardized means is calculated for each sampling period (EPA 1992) to determine if it has crossed the 'decision internal value' (h). If h is exceeded, it can indicate a rapid or

slow rise in concentration at the monitoring well. A gradual increase is indicated when the CUSUM exceeds h and the standardized mean does not exceed the SCL.

Figure 4 illustrates the importance of using the SCL and CUSUM for monitoring lixiviant excursion. The SCL (Z) and CUSUM (C) are plotted for an excursion parameter, a gradual increase in contamination exceeds the CUSUM limit in February of 2002, while the SCL limit is not exceeded until January of 2003. The SCL limit is similar to the CBR's use of 5 standard deviations above the mean for any one sampling event, although EPA recommends 4.5 standard deviations for any one sampling event. Using only the SCL limit allows contamination to migrate beyond the monitoring well for nearly a year before an excursion is declared. Therefore, if the CUSUM is not used with the SCL limit a gradual increase in contamination will not be detected and migration of diluted lixiviant will pass the montoring well without corrective action.

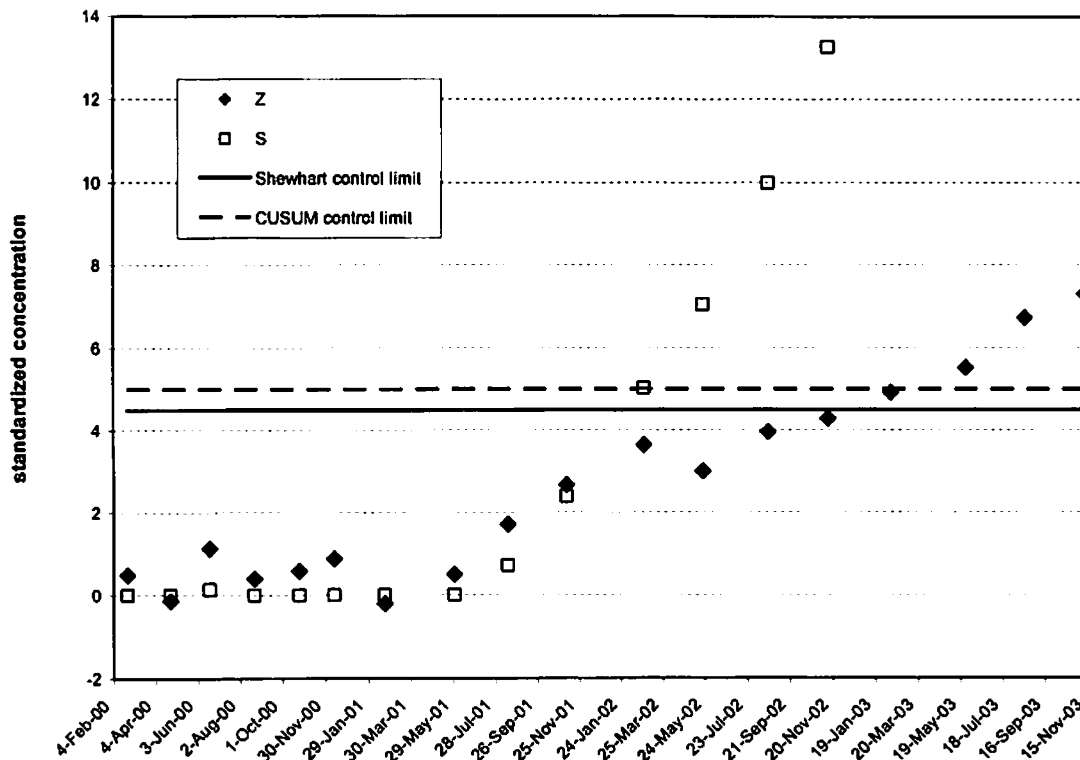

Figure 4. Proper use of a control chart to determine lixiviant excursions.

Geochemical Consulting Services, LLC

Solubility, Speciation, and Reaction-Path Modeling
Groundwater and Soil Geochemistry
Environmental Assessment
Risk Assessment

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Although there was insufficient time to develop a detailed analysis of all the concerns and omissions in the application, I note 46 additional issues that warrant a more detailed evaluation.

1) Section 1.8.1 notes that the only radioactive airborne effluent is radon-222 gas. This is not correct in the strict sense, as the radioactive daughters of radon-222 (Po-218, Pb-214, Bi-214, Po-214, Pb-210, Bi-210, Po-210) form in the radon-22 gas cloud emitted from the facility. The radioactive daughters fallout as the plume drifts downwind, and particulate monitoring downwind should be performed to determine the fallout dose.

2) Section 1.11 notes that a yearly review is done to ensure that proper funds have been set aside for restoration. A key factor in calculating the amount of financial surety is the number of pore volumes of groundwater that must be processed to restore the aquifer to per-mining levels. As pre-mining levels are often biased improperly to high values, the number of pore volumes needed to restore the aquifer is underestimated and insufficient surety is posted.

3) There are no data to support the water quality results in Table 2.2-9. All data must be provided to allow an independent reviewer to derive values presented in the table. Use of the mean implies that the proper statistical test was performed to demonstrate that the data follow a normal or log normal distribution. There is no discussion of the use of statistical distribution tests.

4) Table 2.5-13 summarizes particulate data for the Black Hills and Rapid City, and is used to conclude that there is no problem with particulate matter less than 10 micorns (PM_{10}). This is unacceptable. Site specific data must be collected to demonstrate that the CBR site does not emit PM_{10} that exceeds 150 ug/m^3 (24-hour average) or 50 150 ug/m^3 (annual average).

5) Section 2.6.1.5 notes that the Chadron Sandstone formed as part of a vigorous braided stream system in the early Oligocene. Braided stream systems form a complex assemblage of sediments that consist of channel sands and gravels isolated by sand, silt and clay bank deposits. The primary flow for groundwater is through the channel sands and gravels, and the width of these channels are generally much narrower than the 400-foot spacing of wells in the monitoring ring. Therefore, it is possible that a paleochannel could exist between two monitoring wells and allow pregnant lixiviant to flow past the montoring wells without being detected. There is no discussion on this type of aquifer heterogeneity in Section 2.7.2.3.

6) Figures 2.6-4 through 2.6-11 show the thickness of the Chadron to be 40 to 80-feet thick through most of the mine area. Therefore, it is inappropriate to use a screened interval of 20 feet to sample the groundwater from the ore zone (Table 2.9-3). The entire thickness of the aquifer must be sampled to obtain a representative sample.

7) Tables 2.7-6 through 2.7-16 are not supported by the analytical results used to derive the reported values. See comment 4.

8) Section 2.9 notes that a preoperational monitoring was conducted for nonradiological parameters. This is unacceptable. Uranium and radium must also be considered because exploration holes placed in the ore zone disturb the ore and create a path for oxygen. The disturbance of the ore will expose new uranium mineral surfaces to the groundwater, which will release additional uranium, radium and their progeny. Addition of oxygen to the disturbed region will increase the dissolution of uranium ore minerals.

9) No justification is provided for the location of water-quality wells within the monitoring ring on Figure 2.9-2. Valid statistical methods must be used to locate the systematic or random samples on a grid than covers the entire area enclosed by the monitoring wells.

10) There are no data to support the water quality results in Table 2.9-4. See comment 4. Additionally, if preoperational monitoring was only for nonradiological parameters (see comment 9), when where the samples collected for uranium and radium results that appear in the table?

11) Section 2.9.4 is on surface water quality, but there are no data in the report stream water quality. Surface and buried pipelines that fail catastrophically or slowly leak pregnant lixiviant could contaminate surface water. Pipelines transferring pregnant lixiviant from the well fields to the processing facility are monitored for sudden drop in pressure, which indicates a massive failure and spill. However, small leaks in the buried pipelines, along joints and valves, would not be indicated on the monitor. Therefore, large volumes of pregnant lixiviant could be released to the environment from small leaks over the period of years. Surface waters should be monitored and sampled on a quarterly basis.

12) The end of Section 2.9.4 notes that suspended sediment samples have not been collected since 1982 and there is no plan to collect further samples. This is unacceptable, for reasons noted in comment 11.

13) Soil results in Tables 2.9-10 and 2.9-11 have no results for molybdenum. Molybdenum is known to be concentrated by certain plants and cause problems when livestock ingest the plants containing Mo.

14) On page 3-21 the assumption is made that the aquifer is homogenous and isotropic. This is a poor assumption for fluvial deposits, as there is considerable lateral variability in the grain size (gravel, sand, silt, clay) and preferred flow paths will follow paleochannels.

15) Page 3-32 notes that a risk assessment was performed for the chemical storage facility. There are no assumptions or exposure scenarios discussed to determine if the conclusions are valid.

16) Section 3.3 discusses instrumentation used to monitor the flow out of and into the well fields. There is no detail provided on the pressure drop needed to denote a leak in the piping system transporting pregnant lixiviant. Is a leak of one liter a minute detectable? If so, what pressure drop is associated with such a leak and what is the sensitivity of the system to detect such a drop? If this cannot be detected, there is a potential for a significant amount of contamination to be released over the lifetime of the well field. A one liter per minute leak would result in 1440 liters per day released to the environment.

17) The pond inspection program discussed on page 4-5 does not address air monitoring around the ponds. Radon, mist, and particulate may be mobilized by the wind from the pond and dried margins. Why is air monitoring omitted? What data support such a decision?

18) Page 4-6 notes that if a pond liner leaks, the pond contents will be transferred to another pond. This creates a potential exposure scenario where the contaminated sediments dry out and become airborne by the wind. Air monitoring for particulate and radon is needed around the ponds.

19) Page 4-7 notes that flow-monitoring alarms are activated for a significant piping failure. This implies that a slow leak will not be detected. As noted in comment 16, a slow leak can result in significant contamination of the environment.

20) Page 4-8 (Piping) notes that large leaks would be detected quickly. Again, a small leak could go undetected for years because the piping is buried. This is unacceptable.

21) Page 4-9 notes that the most common surface release is from piping. How is the spill cleaned up? What is done with the contaminated soil?

22) Section 4.2.2.4 (Hazardous Waste) does not mention the arsenic and selenium released from the ore zone. What is the quantity generated and where does it end up in the waste streams?

23) Page 5-15 mentions pond sprays from the enhanced evaporation system. This system has the potential to release mist to the surroundings. See comment 17.

24) Section 5.8 discusses radiation safety controls and monitoring. There is no discussion of air monitoring for radon and daughters downwind of the exhaust vents. What data support such an omission, given hundreds of curies of radon are emitted from this facility.

25) Page 5-28 notes subsurface releases are from ponds and excursions. There can also be subsurface releases from slow leaking pipelines when the leak is too slow to set off the alarm,

26) Section 5.8.7.2 discusses radon monitoring, and notes that 7 locations are monitored. There is no map to show the location of these monitors relative to facilities and downwind direction.

27) Page 5-78 discusses results for air particulate, and notes uranium results are shown on Figs 5.8-18 through 5.8-24. Why are there no displayed results for Ra-226 and Pb-210?

28) Page 5-87 notes that uranium was elevated in the sediment from English Creek. Sediments downstream from the mine areas should be monitored in the future to determine if concentrations increase in the future.

29) The discussion on monitoring well baseline water quality (p. 5-107) indicates the wells are only used to establish excursion limits, which reveals the inadequate approach to establishing baseline in the exempt zone of the aquifer. Monitor wells will reflect the baseline water quality in most of the exempt zone, and should be used to establish baseline in the exempt zone.

30) The discussion on upper control limits and excursion monitoring (p. 5-107) does not cite statistically valid methods for establishing the upper control limits. The use of the noted improper method can result in a large volume of contaminated

groundwater to pass by the monitor wells, as the proposed method only accounts for a rapid increase in contamination, and not a slow increase that is more representative of a migrating plume.

31) The absence of uranium as an indicator of excursion is not justified (p. 5-107). Uranium is highly mobile in the lixiviant and is an excellent indicator of excursions.

32) Section 6.1.3.1 notes that one baseline well per 4 acres is used to establish water quality prior to mining. Are the wells randomly located within each 4 acre zone. If not, why not?

33) Section 6.1.3.2 states that if the baseline concentration exceeds the NDEQ MCL, then the baseline average plus two standard deviations is used to set the restoration goal. What is the justification for this approach? Using the mean and standard deviation is inappropriate unless it can be demonstrated that the data follow a normal or log normal distribution.

34) Analytical data to support the results in Tables 6.1-2 through 6.1-11 are not available to verify that proper statistical methods were used to derive the restoration results.

35) Section 6.1.4 states that Mine Unit 1 was successfully restored to primary or secondary standards. Bicarbonate, sulfate, manganese, selenium, vanadium, uranium and radium were not restored to their primary standard, and there is no summary of secondary standards in Table 6.1-2. What secondary standards apply and why?

36) Section 6.2.3.4 notes that on site burial is possible. If the disposal ponds are to be used as burial sites, will the liners in the system be redesigned to account for permanent disposal? What limits will be placed on the materials that can go into the disposal cell? Will a risk analysis be performed to justify the construction of a disposal cell?

37) Section 6.4.1 gives clean-up criteria for radium and uranium in soil. Why are there no clean-up levels listed for radon decay products (e.g., lead-210), arsenic, molybdenum and selenium?

38) Section 7.6 and 7.12.1.1 discuss air quality impacts. There is no discussion of potential air impacts from contaminated particulate during decommission

activities. The disturbance of contaminated soil during site remediation could suspend contaminants and transport them considerable distances. What type of air monitoring will be performed to ensure that contamination is not spread by air borne dust?

39) Section 7.12.5 discusses air exposure and notes radon and its decay products are the only concern. This is incorrect. Particulate from contaminated soil and mist from the evaporation ponds are also air exposure concerns. Why is there no discussion of these sources?

40) The MILDOS-Area code was used to model the radon dose to receptors. Why are there no input and output files provided to evaluate the model? Tables 7.12-3 through 7.12-7 provide some of the model information. Absent is the wind rose for the area, average wind speeds at 10 and 60 meters, rainfall events and duration, and topographic effects that influence the model results. Also, there is no summary table to compare model results with actual measurements from radon monitors.

41) There is insufficient data provided for the accident scenarios discussed in Section 7.14.5 to properly evaluate the meaning of the stated results.

42) The discussion of economic impacts under Section 8.1.2 notes that failure to renew the license will be detrimental to the economy in the area. However, there is no discussion of the long-term effects of mining. In reality, mining will end and the economy will suffer at some point, and there is little chance for recreation or other industry in an area contaminated by ISL operations. Therefore, the discussion in this section is merely innuendo to intimidate the reader.

43) Section 8.3.1.2 discusses the effectiveness of groundwater restoration as a reason to continue mining. Based on comment 35, one can hardly say the restoration was an overall success. Only by using undefined secondary standards can CBR claim to have restored the groundwater.

44) Section 9.3 notes the groundwater impact is temporary, as restoration returns the groundwater to pre-mining levels. This is simply not true. Restoration to pre-mining levels was not achieved in Mine Unit 1 (comment 35). Secondary standards are not pre-mining levels.

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Environmental Assessment
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45) Section 9.3 also notes radiological impacts will be small because all radioactive wastes will be transported off site. This is a false statement, as comment 36 notes that on site disposal is a possible option.
46) Section 9.4 states there is considerable value offered by CBR to the U.S. energy needs. This implies all the mined uranium is bought and used by the U.S. What assurance is given by CBR that all their mined uranium that is sold on the spot market ends up in the U.S.? Can any ISL operation tell the buyer of their product that the product has to stay in the U.S.?

References

Crow Butte Resources, Inc., 2007, Application for 2007 License Renewal, USNRC Source Materials License SUA-1534, Crow Butte License Area, Crawford, Nebraska.

U.S. Environmental Protection Agency (EPA), 1992. Statistical Analysis of Ground-Water Monitoring Data at RCRA Facilities, Addendum to Interim Final Guidance, Washington, DC.

U.S. Environmental Protection Agency (EPA), 2000a. Guidance for the Data Quality Objective Process – QA/G-4, EPA/600/R-96/055, Washington, DC.

U.S. Environmental Protection Agency (EPA), 2000b. Guidance for the Data Quality Assessment – Practical Methods for Data Analysis - QA/G-9, EPA/600/R-96/084, Washington, DC.

Statistical Analysis of Ground-Water Monitoring Data at RCRA Facilities, Addendum to Interim Final Guidance, Washington, DC.

Pacific Northwest National Laboratory (PNNL), 2007. Visual Sample Plan, Version 5.0 User's Guide, PNNL-16939, Richland, WA.

Sincerely,

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Environmental Assessment
Risk Assessment

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There is an Allen-Bradley PLC-5 based control system in place. However, we have no information about the level of system detail this provides. The system should have a Supervisory Control and Data Acquisition (SCADA) system that provides all water level, well pump and pipe flow telemetry data to one location, linked to a real-time well and pipe flow-modeling system. This would provide the well field operator with the best information to determine what may have caused the excursion. It could also alert the operator to a problem before it becomes an excursion.

Offsite Baseline Water Quality Sampling

The only way to quantify any contamination is to have a baseline for comparison. Historic water quality information from sources such as the USGS should be explored. Existing offsite wells in the vicinity of CBR and wells along the White River alluvium should be sampled for pertinent water quality parameters to establish this baseline. In areas with no existing wells, monitor wells should be installed. In addition, surface water quality sampling sites should be established along the White River and its tributaries.

Characterization of Contamination Pathways/Offsite Aquifer Parameters

As part of any site characterization, aquifer parameters such as transmissivity and saturated thickness should be established. Potentiometric surfaces should be mapped. This will likely require test holes, monitor wells, and pumping tests. Surface and borehole geophysical techniques and hydrophysics should be considered to characterize the system in sufficient detail.

Surface geophysical techniques can help define the geometry on the depositional environments of the White River alluvium and underlying units. Borehole geophysics can provide information about boundaries and preferential pathways. Hydrophysical examinations of the subsurface can characterize things like fracture flow to quantify secondary hydraulic conductivity. Many new techniques have emerged in recent years. For example, scanning colloidal boroscope flow meters can track naturally-occurring colloidal-sized particles in groundwater, provide very accurate measurements of speed and direction of natural flows through boreholes, aiding in identification of preferential pathways.

Depending on other investigation results, a numerical groundwater flow and contaminant transport model such as MODFLOW/MT3DMS may be needed to quantify the extent of the contamination. This type of modeling effort would require extensive field data for calibration and verification.

White River Contamination

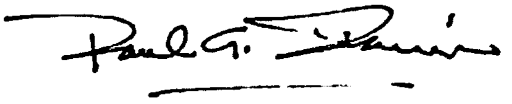
In reference to statements from Dr. Hannan LaGarry, we agree with his assessment that an examination of the extent of contamination of the White River alluvium is warranted. Many of his suggestions can be augmented by the geophysical and hydrophysical techniques previously mentioned. We agree that the subsurface should be fully characterized in as much detail as

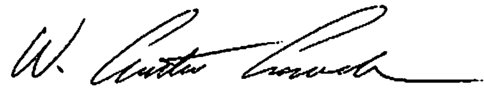
possible, using a Geographic Information System (GIS). Subsurface mapping in three dimensions will substantially contribute to an overall understanding of this system.

As mentioned previously, a re-examination of select boreholes could employ newer techniques to understand the overall geologic setting as well as contaminant fate and transport. Areas of concern for data gaps could be refined through the use of these newer techniques.

We understand the concerns of down-gradient water users, such as the Towns of Crawford, Chadron and Pine Ridge. The extent of contamination is unknown at this stage. Any plan for sampling and characterization should be flexible until the full extent of the problem is better understood. Any monitor wells installed should be constructed so as to be considered permanent, so that extended monitoring may be conducted indefinitely.

Respectfully,





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J·R ENGINEERING
A Westrian Company

July 28, 2008

David Cory Frankel
ARM Aligning for Responsible Mining
P.O. Box 3014
Pine Ridge, South Dakota 57770

Re: Summary of Recommendations and Opinions on CBR

Dear Mr. Frankel:

We have conducted a limited review of the 2007 License Renewal Application for Crow Butte Resources (CBR). Overall, we found it to be a professionally written document, with a large amount of useful information.

It is our understanding that there has been some offsite contamination as a result of mining operations at the CBR site, and that there is a likelihood of further contamination of the alluvium along the White River from these mining operations. It certainly should be the goal of all concerned parties that any further migration of contaminants off site be stopped as soon as possible. This requires an understanding of the mining operations, the local geology, and how the contaminants got released. It should also be the goal to understand the nature and extent of the contaminated area, so that informed decisions can be made about any mitigation.

To that end, we make the following recommendations:

<u>Better Monitoring and Response to Excursions</u>

Monitor wells at the CBR site appear to be only screened in the ore-bearing part of the Chadron formation. There should be additional monitor wells that are completed in all of the water bearing formations above the Pierre Shale. In order to prevent cross contamination of aquifers, and to establish which aquifer is indicating an excursion, any one monitor well should be sealed and screened in only one aquifer. For any one location, this would require a set of wells independently monitoring the Chadron, Brule, and alluvium.

We understand that there are over 5000 wells at the CBR site. We also have been informed that anytime a lixiviant excursion is detected in the monitoring system, a person has to physically go to the well field and make some adjustment, based on that person's judgment of the situation. The complex nature of this well system suggests that this method is likely to be error prone.

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ATTACHED are studies referenced in the Oglala Sioux Tribe's Petition for Intervention.

Testimony before the Committee on Oversight and Government Reform

Congress of the United States

House of Representatives

October 23, 2007

By Doug Brugge, PhD, MS

Good morning/afternoon Chairman Waxman and members of the committee. My name is Doug Brugge, I have a PhD in cellular and developmental biology from Harvard University and an MS in industrial hygiene from the Harvard School of Public Health. I am currently associate professor in the department of public health and family medicine at Tufts University School of Medicine. I also direct the Tufts Community Research Center. I have over 20 academic publications about uranium and the Navajo people, including a 2006 book that I co-authored, entitled The Navajo People and Uranium Mining. I have studied the Navajo people in part because they are facing a crisis in uranium contamination.

Appearing before this congressional hearing today reminds me of the long history of such hearings, beginning in the 1960s and continuing through the 1970s, 80s and 90s, that sought and eventually achieved a semblance of compensation for Navajo and other uranium miners. I am deeply saddened by the fact that so little has been accomplished over those decades to eliminate the health hazards faced by the enormous quantities of uranium waste on the Navajo reservation. There has been too little research on the health impacts of uranium mining in Navajo communities. The one study underway, for example, will mostly address kidney disease and not birth defects or cancer. Today as we begin the public process of addressing community exposures, I can only hope that the path is far shorter than the one traveled by the uranium miners and their families.

I will now spend a few moments describing the hazards faced by the Navajos today. Clearly, uranium ore is a toxic brew of numerous nasty hazardous materials. Uranium, itself highly toxic, gives rise to a series of other radioactive decay elements that are found in raw, natural ore. Most significant among these are radium and thorium, both of which are highly radioactive. When radium decays it produces radon gas, a potent toxicant. Because it is a gas that becomes

Island release, a dam holding back a tailings lagoon maintained by United Nuclear Corporation failed, sending 94 million gallons of radioactive and acidic wastewater and 1,100 tons of toxic and radioactive mill waste into the Puerco River. This release, which was substantially larger than the release at TMI, flowed into a low-income, largely Native American community. This incident has been virtually ignored in the press and scientific literature.

For the people in Church Rock and other Navajo communities contaminated for decades with uranium ore tailings there are no "good" options, too much harm has already been done. But there are ways that we can gradually make things better so that maybe the children and the grandchildren of the Navajo uranium miners are not still grappling with this toxic legacy. A good start would be to provide sufficient resources to secure or remove contamination at these hazardous waste sites and to do so in a manner that prevents additional exposure to nearby residents. And Congress must fund the Navajo Nation and federal health agencies to provide resources for health studies among the tens of thousands of Navajo community members who still live next to abandoned mines and-or who were exposed to uranium from the contaminated dusts brought home by their working relatives.

I leave you to ponder a simple observation about this egregious situation: As terrible as the health effects that we know arise from toxins in uranium tailings, there are almost certainly additional ways that the health of Navajo people living near uranium mill and mine waste has been affected. If we are to understand the full extent of this injustice, we will also need additional health studies.

airborne, when radon decays it transforms into a series of highly radioactive "radon daughters" that can lodge in the lungs.

The primary heavy metal toxicants in uranium ore include uranium itself and arsenic, as well as vanadium and manganese. During the first phase of processing uranium, most of the uranium is removed, leaving behind mill tailings which retain most of the other toxic contaminants from the ore. The milling of uranium is an industrial process that involves crushing and grinding of the rock and the addition of acids and organic solvents to facilitate concentration and removal of the uranium. Hence, uranium mill tailings and mill tailings effluent are not only highly radioactive, but they are acutely hazardous.

The health effects of uranium and its associated radioactive decay products and heavy metals that rise to the level of proven or near-proven causal links include:

1) Radon, which causes lung cancer and in fact, it is the primary source of lung cancer among Navajo uranium miners;
2) Uranium, which as a heavy metal causes damage to the kidneys and birth defects ;
3) Radium, which causes bone cancer, cancer of the nasal sinuses and mastoid air cells and leukemia; and
4) Arsenic, which causes lung and skin cancer, as well as neurotoxicity, hyperpigmentation and hyperkeratosis of the skin.

There are may also be many other negative health effects from exposure to uranium and its byproducts. In short, there is a clear causal link between uranium exposure and human health. The Navajos continually exposed to uranium and its byproducts even today face grave threats to their health.

I would like to conclude with some observations about the Navajo community of Church Rock, both historical and present day. Church Rock is located outside of Gallup, New Mexico, in the Navajo Nation. The Church Rock tailings spill remains the largest industrial release of radioactive wastes in the history of the United States. In 1979, only months after the Three Mile

Cancer Disparities Research Partnership in Lakota Country: Clinical Trials, Patient Services, and Community Education for the Oglala, Rosebud, and Cheyenne River Sioux Tribes

| Deborah Rogers, MS, and Daniel G. Petereit, MD

Native Americans served by the Aberdeen, Billings, and Bemidji areas of the Indian Health Service (IHS) have a cancer mortality rate approximately 40% higher than that of the overall US population. The National Cancer Institute has funded Rapid City Regional Hospital to provide clinical trials, behavioral research, a genetic protocol, patient navigator services (assisting patients with health care coordination and financial issues and helping them to understand their options), and community education for members of 3 western South Dakota tribes.

Challenges faced by the project included obtaining multiple approvals from 3 tribes, 4 IHS facilities, and 5 institutional review boards; travel distances; lack of screening; red tape of referrals; and refusal by some payers to cover clinical trials. Building trust through ongoing communication and community presence is key to a successful project.

IN THE UNITED STATES, various subpopulations experience different rates of cancer detection, treatment, participation in clinical trials, and outcomes.[1–4] In particular, the population of Native Americans served by the 10-state Billings, Aberdeen, and Bemidji service areas of the Indian Health Service (IHS) suffers from a cancer mortality rate approximately 40% higher than that of the overall US population.[5] Researchers from the IHS analyzed cancer mortality data from the death certificate database of the National Center for Health Statistics, which were adjusted for racial miscategorization and the age structure of the population, and then summarized the results for 1994 through 1998. Although their rates of breast cancer mortality were approximately 15% lower than for Whites, Native Americans in this 10-state Northern Plains region had significantly higher average annual age-adjusted mortality rates for colorectal cancer (58% higher), lung cancer (62%), cervical cancer (79%), and prostate cancer (49%).[5]

Rapid City Regional Hospital, in the Black Hills of western South Dakota, provides secondary and tertiary cancer care for an estimated 60000 Native Americans living within a 200-mile radius of Rapid City (Figure 1). Most of these tribal members live in the IHS Aberdeen Area (North Dakota, South Dakota, Iowa, and Nebraska). In 1996 through 1998, the life expectancy at birth (both genders) for Native Americans in the Aberdeen Area was 65.4 years, compared with 70.6 years for all IHS areas (1996–1998) and 76.5 years for the general US population (1997).[6]

The local population served by Rapid City Regional Hospital is growing very rapidly, with nearly 50% of the population aged younger than 18 years, according to data from the 2000 US Census. In 1996 through 1998, the leading causes of death in the Aberdeen Area were diseases of the heart (21% of deaths), malignant neoplasms (15%), unintentional injuries (14%), diabetes mellitus (8%), and chronic liver disease and cirrhosis (6%).[6]

A retrospective chart review was performed for 93 Native American radiation therapy patients treated at the hospital's Cancer Care Institute between January 1998 and October 2002.[7] The median one-way distance patients traveled was 109 miles (ranging from 5 to 215 miles). Thirty-seven percent of Native American patients traveled at least 150 miles each way. Of 61 Native American patients treated with curative intent, 28% had treatment delays (i.e., missed days) of 6 or more days and 15% had delays of 11 or more days. Thirty of the patients (half) experienced grade 2 radiation treatment toxicities, and 10 had grade 3 radiation treatment toxicities.

Statistics from Rapid City Regional Hospital's Tumor Registry (1990–2000) indicate that Native Americans are more likely than other patients to present with advanced (stage III or IV) disease, which leads to lower survival rates (Table 1).[7] For colorectal, breast, prostate, cervical, and lung cancer, approximately 50% of Native Americans arrived at the hospital with advanced cancer, as opposed to 36% of non–Native Americans.

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Uranyl acetate induces *hprt* mutations and uranium–DNA adducts in Chinese hamster ovary EM9 cells

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Questions about possible adverse health effects from exposures to uranium have arisen as a result of uranium mining, residual mine tailings and use of depleted uranium in the military. The purpose of the current study was to measure the toxicity of depleted uranium as uranyl acetate (UA) in mammalian cells. The activity of UA in the parental CHO AA8 line was compared with that in the XRCC1-deficient CHO EM9 line. Cytotoxicity was measured by clonogenic survival. A dose of 200 μM UA over 24 h produced 3.1-fold greater cell death in the CHO EM9 than the CHO AA8 line, and a dose of 300 μM was 1.7-fold more cytotoxic. Mutagenicity at the hypoxanthine (guanine) phosphoribosyltransferase (*hprt*) locus was measured by selection with 6-thioguanine. A dose of 200 μM UA produced ~5-fold higher averaged induced mutant frequency in the CHO EM9 line relative to the CHO AA8 line. The generation of DNA strand breaks was measured by the alkaline comet assay at 40 min and 24 h exposures. DNA strand breaks were detected in both lines; however a dose response may have been masked by U–DNA adducts or crosslinks. Uranium–DNA adducts were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) at 24 and 48 h exposures. A maximum adduct level of 8 U atoms/10^3 DNA-P for the 300 μM dose was found in the EM9 line after 48 h. This is the first report of the formation of uranium–DNA adducts and mutations in mammalian cells after direct exposure to a depleted uranium compound. Data suggest that uranium could be chemically genotoxic and mutagenic through the formation of strand breaks and covalent U–DNA adducts. Thus the health risks for uranium exposure could go beyond those for radiation exposure.

Introduction

Questions about possible adverse health effects from environmental and occupational exposures to uranium have arisen as a result of uranium mining, residual mine tailings, and the use of depleted uranium in the military. Depleted uranium is uranium that has higher levels of ^{238}U and lower levels of ^{235}U and ^{234}U relative to natural uranium. Over half of the US uranium reserves are believed to exist in the Four Corners area of the Southwestern United States (1). It is estimated that over 300 tons of depleted uranium were used during Gulf War I (2), but estimates for Gulf War II have not yet been reported. The impact of these high levels of environmental uranium on exposed populations is of growing concern.

The adverse health effects from occupational and experimental uranium exposures that have been established most significantly include lung cancer, from exposure to ^{222}radon that is produced through the radioactive decay of ^{238}U (3,4), and chemically induced kidney toxicity (5); however, bladder damage (6), birth defects (7) and chromosomal aberrations (8) have also been reported. Thorough epidemiological data for health effects from either environmental exposures to uranium tailings or military exposures to depleted uranium are currently lacking due to insufficient study of both Native American populations other than miners, and the short time span since initial military exposures to DU weapons and munitions. However, evaluation of DU-exposed veterans is in progress (9).

Previous work has shown that depleted uranium as uranyl acetate (UA) produced DNA strand breaks in the presence of vitamin C, which suggested a chemical rather than radiological mechanism (10). The purpose of the current study was to measure the potential for depleted uranium as UA to be toxic in mammalian cells. Depleted uranium was used because, besides being the commercially available form of uranium, it provides less likelihood for chemical effects to be masked by radioactivity. A soluble form of U(VI) was tested here because of an interest in environmental exposure through drinking water; however, it is not assumed that insoluble uranium provides no risk environmentally. Based on previous work *in vitro* (10) it was expected that DNA strand breaks may be formed in cultured cells, and it was presumed that if strand breaks were occurring then those lesions could be relatively easily repaired. For this reason experiments were carried out in both the parental CHO AA8 line and the CHO EM9 line, which has reduced levels of the XRCC1-DNA ligase III complex (11), and is therefore sensitive to DNA strand breaks (12). It was hypothesized that if UA caused direct DNA damage in CHO cells then it should be more cytotoxic and mutagenic in the repair-deficient EM9 line than the parental AA8 line. Results supported these hypotheses; however, the DNA damage produced in CHO cells was not limited to strand breaks, but included U–DNA adducts.

Materials and methods

Reagents and chemicals

Depleted uranium as uranyl acetate dihydrate [6159-44-0] (UA), with a U^{234}/U^{238} activity ratio of 0.12 (10), was obtained from Spectrum Chemical Mfg Corp. (Gardena, CA). 2-Amino-6-mercaptopurine (6-thioguanine) [154-42-7] (Sigma Chemical Co., St Louis, MO) was used as received.

General cell culture conditions

Chinese hamster ovary AA8 and EM9 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells at passage 3 were thawed from cryopreservation, cultured in αMEM (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), antibiotic/antimycotic

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(100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B) (Sigma) and 1 mM glutamine (Gibco-BRL, Rockville, MD). Cells were maintained at 37°C in a 5% CO_2/air humidified incubator calibrated with a Fryrite analyzer (Bacharach Co., Pittsburgh, PA).

Cytotoxicity measurements

Cytotoxicity, as decreased cell survival, was determined by measuring colony-forming ability in the CHO AA8 and CHO EM9 lines. Cytotoxicity measurements were carried out after incubation with UA for 24 h. Cells were seeded at 8×10^5 cells per 100 mm plate, allowed to adhere for over ~20 h, and treated with sterile-filtered aqueous solutions of UA (0–300 μM) for 24 h. Upon completion of exposure, cells were trypsinized, quantified on a Z1 Coulter Particle Counter (Beckman Coulter, Inc., Miami, FL) and reseeded at 200 cells (AA8) or 300 cells (EM9) per 60 mm dish in quadruplicate. After 7 days all dishes were stained with crystal violet and the colonies were counted. Cell survival was calculated as percent colonies in treated dishes relative to untreated controls. Plating efficiency was 86% for the AA8 line and 56% for the EM9 line.

Mutagenicity measurements

The HPRT assay was carried out following published procedures (13) with minor modifications. Cells were exposed to UA at 100–300 μM for 24 h as described above. Cells were harvested and analyzed for cell survival with a 7 day colony formation assay as described above. From the same cell populations harvested for cell survival measurements after 24 h treatment times, approximately 1×10^6 cells were reseeded in 100 mm dishes and that amount was passaged every 3 days until day 9 post-treatment. This expression time was found in previous studies (14) to be optimum (data not shown), and is consistent with recommendations for this assay (13). After this total 10-day expression time cells were seeded again for colony-forming ability as described above, and 2.5×10^5 cells were seeded in quadruplicate in 100 mm dishes and incubated in medium containing 11 μg/ml of 6-thioguanine (6-TG) for 7–8 days for mutant selection. Data are expressed as mutants per 10^6 surviving cells, calculated from the observed 6-TG-resistant colonies and the 10-day clonogenic values. Average induced mutant frequency and average mutant increase above background were calculated from the differences and ratios of individual experiments. Experiments were repeated 4–7 times.

Single cell gel electrophoresis (comet assay)

The ability of UA to produce DNA strand breaks in CHO AA8 and CHO EM9 cells was measured by the alkaline comet assay following recommended procedures (15,16). Briefly, CHO AA8 or CHO EM9 cells were seeded at 8×10^5 cells/ml and exposed to UA after cell attachment. Cells were treated with sterile aqueous solutions of 50–300 μM UA for 40 min or for 24 h at 37°C. As a positive control for strand breaks and oxidative damage, cells were treated with 40 or 80 μM H_2O_2 for 40 min at 37°C. Untreated cells served as a negative control. Cells were harvested by scraping in dim light, pelleted and placed on ice. Microgels were prepared in duplicate on MGE slides (Eric Scientific Inc., Portsmouth, NH) in four layers as recommended (16). All slides were subjected to lysis solution (2.5 M NaCl, 100 mM EDTA tetrasodium salt, 10 mM Tris, pH 10, 1% sodium lauroyl sarcosine, 1% Triton X-100) for 2 h at 4°C.

One set of duplicate slides for each dose was incubated with *Escherichia coli* formamidopyrimidine-DNA glycosylase (FPG) using the FLARE Assay kit (Trevigen, Inc., Gaithersburg, MD) after the 2 h lysis step, in order to detect the presence of oxidative damage. Slides were rinsed in 1× FLARE buffer for 15 min, and the FPG enzyme, at 50 U in 200 μl, was added to the slides for 30 min at 37°C. These slides were then combined with other duplicate slides for the remainder of the assay.

All slides were then subjected to unwinding in alkaline buffer (300 mM NaOH, 1 mM EDTA, 0.2% DMSO, 0.1% 8-hydroxyquinoline, pH >13) for 20 min, followed by electrophoresis for 15 min on a horizontal electrophoresis unit (MGE, Techniport, Inc.) at 250 mA and 4°C with buffer recirculation of 100 ml/min.

After electrophoresis, slides were neutralized in 1 M ammonium acetate in ethanol for 15 min at ambient temperature, followed by incubation in 1 mg/ml spermine in 66% ethanol for 15 min at ambient temperature. Slides were air-dried in the dark before staining. Slides were prestained for 1 min with a 60 μl volume of 5% sucrose and 1 mM monosodium phosphate. Slides were stained 30 min prior to analysis with 200 μl of a 1:10 000 dilution of SYBR® Green (Molecular Probes, Eugene, OR) followed by 50 μl of Vectashield (Vector laboratories, Inc., Burlingame, CA).

Slides were analyzed for DNA damage on an Olympus BX51 epifluorescence microscope equipped with an LAI Comet Assay Analysis System (Loats Associates, Inc., Westminster, MD) at 40× magnification. Tail moment (tail length × percentage of DNA in tail) was measured in 50 cells for each treatment, positive and negative controls, and independent experiments were repeated in triplicate or quadruplicate. The average of 50 cells was calculated for each treatment, and reported data represent the mean ± SEM of the individual averages of the 3–4 independent experiments.

Measurement of uranium/DNA-P binding by ICP-OES

CHO AA8 or CHO EM9 cells were seeded in duplicate 100 mm dishes at 5×10^5 or 7×10^5 cells per plate, respectively, and were either allowed to grow for 48 h and treated with UA at final concentrations of 0–300 μM for 24 h or were allowed to grow for 24 h and treated with 0–300 μM UA for 48 h. At 5 min before harvesting one set of duplicate dishes was treated with 0–300 μM UA to serve as a 'zero time point control' to measure background or membrane-bound uranium that could be carried along in harvested cells to artificially interact with DNA during DNA extraction. Values of U-DNA for zero time points were not statistically different from untreated cells (data not shown). Cells were washed three times with PBS, harvested with trypsin, and cell suspensions from duplicate treatments were combined, pelleted and stored at −4°C until DNA was extracted. Cells were subsequently thawed and lysed in 10 mM Tris, 5 mM EDTA, 5% SDS, 0.2 M NaCl. RNA was removed by incubating samples in lysis buffer containing 2 U of pancreatic RNase A (Sigma) at 37°C for 30 min. DNA was extracted with 25:24:1 phenol:$CHCl_3$:isoamyl alcohol, separated by centrifugation in Light Phase Lock Gel™ tubes, twice precipitated with isopropyl alcohol, washed with 75% and 100% ethanol, air-dried, and digested in 200 μl of 20% HNO_3 and 50 μl of 30% H_2O_2 by heating for 1 h at 80°C. Samples were then diluted to a final volume of 3 ml containing 0.1 p.p.m. ytterium as an internal standard. The digested samples were then assayed for uranium and phosphorous on a PerkinElmer Optima 4300DV inductively coupled plasma optical emission spectrometer (ICP-OES) with a meinhard nebulizer and a cyclonic spray chamber. The plasma operated at 1300 W with a sample introduction rate of 1.50 ml/min. The plasma, auxiliary, and nebulizer flow rates were 15, 0.2, and 0.80 l/min, respectively. The emission wavelengths used for uranium and phosphorous were 385.958 and 213.617 nm, respectively. The metal-nucleotide binding ratios were calculated as the moles of uranium per mole of phosphorous in the sample. The limits of detection were 1.805 p.p.b. (7.58×10^{-9} M) for uranium, and 9.322 p.p.b. (3.01×10^{-7} M) for phosphorous. The limits of quantitation were 6.402 ppb (2.69×10^{-8} M) for uranium, and 20.71 ppb (6.69×10^{-7} M) for phosphorous.

Statistics

Statistical significance was evaluated by ANOVA using the Tukey *post hoc* test. Differences were considered significant at $P < 0.05$. Statistical outliers were verified by Grubbs' test (extreme studentized deviate method), $P < 0.05$.

Results

It was initially hypothesized that if UA caused DNA strand breaks *in vitro* (10) then UA should be more toxic in the strand break-sensitive CHO EM9 line than in the parental CHO AA8 line. This hypothesis was tested by measuring the cytotoxicity and mutagenicity of UA in CHO AA8 and EM9 cells.

The cytotoxicity of UA was measured by a colony formation assay. Cell survival of UA decreased with increasing doses in both CHO AA8 and CHO EM9 cells after 24 h exposures (Figure 1). UA was more cytotoxic in the EM9 line than in

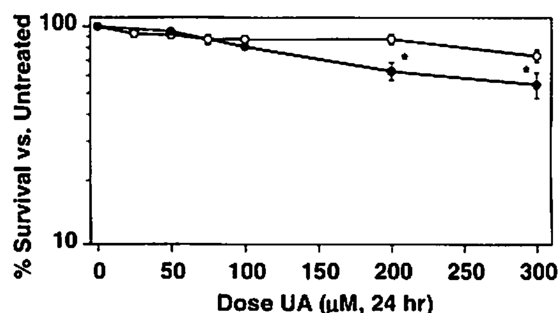

Fig. 1. Cytotoxicity of UA in CHO-AA8 (open circles) and CHO-EM9 (closed circles) cells after 24 h exposures. Cells were treated and assayed for 7–8 day colony formation as described in the text. Data represent mean ± SEM for n = 8–11 independent experiments. The asterisk indicates statistical significance between equivalent doses in the AA8 and EM9 lines at $P < 0.05$.

Table I. Cell survival and mutation induction in CHO AA8 and CHO EM9 cells treated with UA for 24 h

Treatment (24 h)	Survival[a] (10 Days)		Average mutants per 10^6 survivors		AIMF[b]		Average mutant increase above background[c]	
	AA8	EM9	AA8	EM9	AA8	EM9	AA8	EM9
Untreated	100 ± 0	100 ± 0	5.1	5.3	(0)	(0)	(1.0)	(1.0)
100 μM UA	99 ± 3.2	85 ± 4.8	9.8	16.2	4.5	11.0	5.7	4.8
200 μM UA	98 ± 3.1	90 ± 1.9	11.8	36.6	6.6	31.3[d]	4.0	7.8
300 μM UA	102 ± 3.0	85 ± 2.6	10.7	27.2	5.9	22.0	2.3	4.7

[a]Colony formation measured after 24 h treatments plus 9 day growth time for expression of mutant phenotype. Colony formation after 24 h exposures is shown in Figure 1.
[b]Treatment MF–control MF per 10^6 viable cells averaged from differences in individual experiments.
[c]Treatment MF/control MF averaged from ratios in individual experiments.
[d]Significantly different from equivalent dose in AA8 cells, $P < 0.05$.

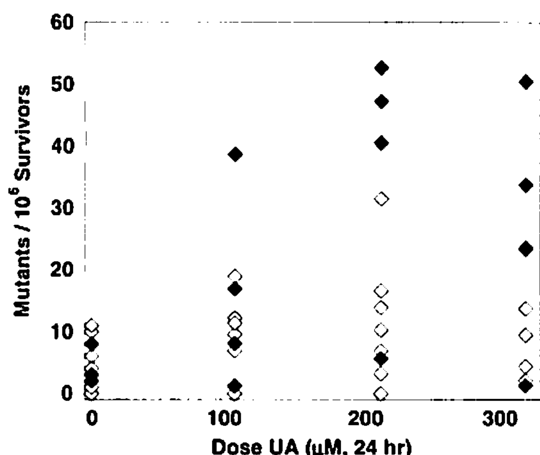

Fig. 2. 6-Thioguanine-resistant cells obtained after 24 h exposure of CHO-AA8 (open diamonds) and CHO-EM9 (closed diamonds) cells to UA. Methods are described in the text. Each data point refers to an individual experiment.

the AA8 line, with the 200 μM dose producing 12 and 37% cell death in the AA8 and EM9 lines, respectively, and the 300 μM dose producing 26 and 45% cell death in the AA8 and EM9 lines, respectively. The observation that UA was more cytotoxic in the repair-deficient EM9 line than the parental AA8 line supported the initial hypothesis and was interpreted to suggest that UA caused DNA damage in CHO cells.

The mutagenicity of UA was measured at the *hprt* locus by a selection of cells resistant to 6-thioguanine (6-TG). After 24 h treatment and a 9 day recovery time to allow for expression of the mutant phenotype, survival of UA-treated cells recovered to ≥98% in the AA8 line and ≥85% in the EM9 line (Table I). UA was weakly mutagenic in the two CHO lines (Figure 2). The untreated AA8 cells averaged 5.1 spontaneous mutations (range 0–11), and the EM9 line averaged 5.3 spontaneous mutations (range 2–8) (Table I). The highest induced mutant frequency was observed in the EM9 line for the 200 μM and 300 μM doses, at 31.3 and 22, respectively, which were ~5-fold and ~4-fold higher than the frequency observed for equivalent doses in the AA8 line. These data were consistent with the interpretation that if UA caused direct DNA damage in CHO cells, then at least some, but perhaps not all, of the resulting DNA lesions were mutagenic.

It was hypothesized that if UA was mutagenic in repair-deficient cells then DNA damage should be occurring either by direct uranium–DNA interactions or by generation of reactive oxygen species. This hypothesis was tested by measuring DNA damage as strand breaks, oxidative damage and uranium–DNA adducts in CHO EM9 and AA8 cells exposed to UA.

The presence of DNA strand breaks was measured by the alkaline comet assay in cells exposed to 50–300 μM UA for 40 min and 24 h. Exposure to 40 and 80 μM H_2O_2 for 40 min at 37°C served as the positive control. Hydrogen peroxide showed an increase in tail moment with increasing dose in both cell lines (Figure 3A and B). In the AA8 line post-treatment with FPG significantly increased the tail moment for both H_2O_2 doses ($P \leq 0.01$), suggesting that oxidative damage was present, as expected (Figure 3A). However, FPG did not produce a significant increase in H_2O_2-induced tail moment in the EM9 line (Figure 3B). The mean tail moments for CHO EM9 cells exposed to 40 or 80 μM H_2O_2 were slightly lower than those in the AA8 line. The mean tail moments for CHO EM9 cells exposed to H_2O_2 with FPG post-treatment were significantly lower than equivalent exposures in the AA8 line for both 40 and 80 μM doses ($P < 0.02$). These results are consistent with a study that found less H_2O_2-induced DNA strand breaks in the EM9 line relative to the AA8 line (17).

Exposure to UA at 40 min resulted in significant increases in tail moments relative to untreated controls for all doses (50–300 μM) in both cell lines ($P < 0.05$); however, no dose response was apparent in either cell line (Figure 3A and B). There was no significant difference in tail moments between lines for equivalent UA doses, nor did FPG have a significant effect on increasing tail moment for any dose of UA in either line.

Exposure to UA at 24 h in the AA8 and EM9 lines produced similar increases in tail moment relative to untreated controls and a similar lack of clear dose response in both lines (Figure 3C and D). Treatment with UA plus post-treatment exposure to FPG yielded no significant increase in tail moments relative to treatments with UA alone in either cell line. These results for both 40 min and 24 h exposures could mean that UA did not produce any formamidopyrimidines, and could thus be interpreted to suggest that oxidative damage did not occur in UA-treated cells under these conditions. Data could also be interpreted to suggest that DNA strand breaks did occur in UA-exposed cells; however, the dose response in the comet assay could have been masked by the presence of another lesion, for example DNA crosslinks (*vide infra*).

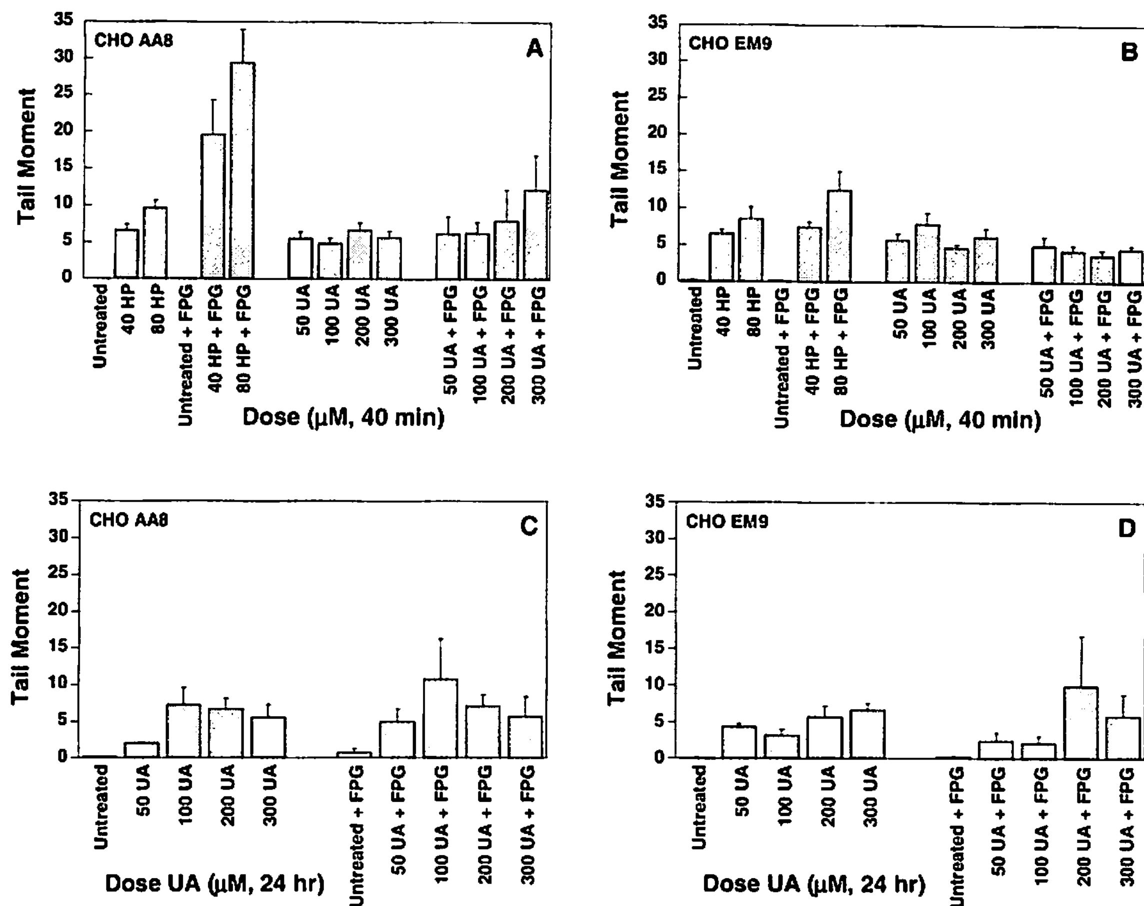

Fig. 3. Analysis of DNA damage induced by UA and H_2O_2 by the comet assay. (A) CHO AA8 cells exposed to H_2O_2 or UA for 40 min at 37°C. (B) CHO EM9 cells exposed to H_2O_2 or UA for 40 min at 37°C. (C) CHO AA8 cells exposed to UA for 24 h. (D) CHO EM9 cells exposed to UA for 24 h. Cells were analyzed for strand breaks, or were post-treated with FPG and analyzed for oxidative damage. Data represent mean tail moment ± SEM for $n = 3$–4 independent experiments. Within each experiment 50 cells were scored for each dose.

Uranium is a metal that forms bonds with biological molecules; thus general uranium-DNA adducts could represent another potential class of DNA lesions. The ability of UA to produce uranium–DNA adducts was therefore measured by ICP-OES in both cell lines. Cells were exposed to 0–300 μM UA for 24 and 48 h. DNA was extracted and precipitated from washed, harvested cells. After digestion in HNO_3/H_2O_2, concentrations of uranium and phosphorous were measured by ICP-OES and ratios of uranium to DNA-P were calculated. Experiments were carried out with and without RNaseA to compare uranium binding to DNA versus total nucleic acid. Data showed that uranium–DNA adducts existed on the order of a few U atoms per thousand nucleotides, and increased with increasing dose and increasing exposure time for 24 and 48 h treatments in both cell lines (Figure 4).

The effect of RNase A on adduct levels was only significant in the AA8 line at the highest dose tested. In the AA8 line, at the 24 h exposure there were 2.4-fold more ($P < 0.0001$) U–DNA adducts in samples exposed to RNase A relative to samples for which RNA was not degraded (Figure 4). At the 48 h exposure there were 2.5-fold more ($P < 0.001$) U–DNA adducts in RNase-treated samples. In the EM9 line there was no difference between uranium adducts in DNA versus total nucleic acid.

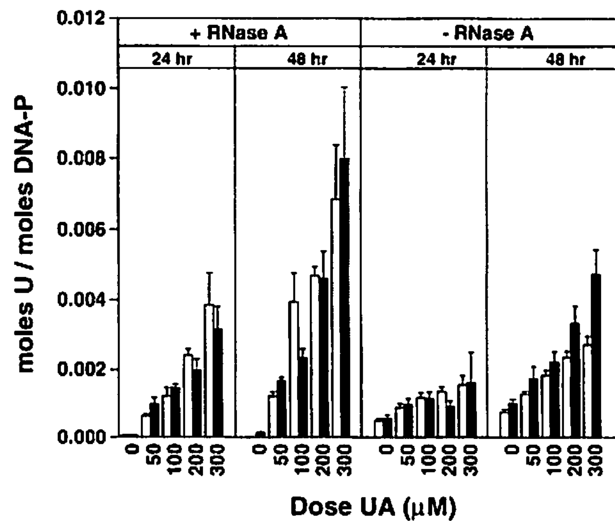

Fig. 4. Measurement of uranium–DNA binding in CHO AA8 (open bars) versus CHO EM9 (closed bars) cells exposed to UA for 24 or 48 h by ICP-OES. Uranium was found bonded to DNA after incubation with 2U RNase A (+RNase A) and without (−RNase A). Data represent mean ± SEM for $n = 4$–9 experiments.

In general, there was no significant difference in U–DNA adducts between the AA8 and EM9 lines at either exposure times. In samples not exposed to RNase A, the EM9 line showed 1.7-fold higher U-nucleic acid adducts than the AA8 line after 48 h; however, this difference was not seen after the 24 h exposure, nor was it evident in samples exposed to RNase A.

Discussion

Measurements of UA-induced DNA toxicity were carried out in both the repair-proficient CHO AA8 line and the repair-deficient CHO EM9 line. The compromised DNA repair in the EM9 line is due to expression of 4-fold lower DNA ligase IIIα and ∼10-fold lower X-ray repair cross-complementing gene I protein (XRCC1) relative to the parental line (11). Ligase IIIα catalyzes the rejoining of the DNA phosphodiester backbone and is thus involved in base excision repair and repair of DNA strand breaks (18). The XRCC1 protein has no catalytic activity but forms complexes with ligase IIIα, as well as with poly(ADP-ribose) polymerase 1 (PARP-1), PARP-2 and several other DNA repair proteins (19). The observation that UA was more cytotoxic in the CHO EM9 line than the CHO AA8 line, coupled with the assumption that the only difference between the two lines was their ability to repair DNA damage, was inferred to suggest that UA caused direct DNA damage that was differentially repaired in these two lines.

The lack of a significant difference between the two cell lines in terms of DNA strand break production was unexpected; however, strand breaks were estimated by mean tail moment in the comet assay, and the presence of other forms of damage may have interfered in the migration of DNA in that assay. For example, if U–DNA adducts existed in the form of crosslinks, or if crosslinks that did not contain uranium were present, those lesions could decrease tail migration, as has been observed for platinum and other crosslinking agents (20).

Comparisons can also be made between the two lines in terms of cytotoxicity. The 2- to 3-fold increased cytotoxicity of UA in the EM9 versus AA8 lines places UA in the same category as chemicals known to cause DNA strand breaks and DNA crosslinks, for example H_2O_2 (17), chromate and mercuric chloride (21), UVA light (22) and near visible and blue light (23). The observation that monofunctional alkylating agents ethyl methanesulfonate and methyl methanesulfonate were >10-fold more cytotoxic in the EM9 versus AA8 lines (24) is consistent with the interpretation that UA may not form monofunctional adducts or apurinic/apyrimidinic (AP) sites as predominant lesions.

The current observation of UA-induced *hprt* mutations is consistent with previous reports of soluble uranyl causing cellular and genetic damages in mammalian cells. Uranyl nitrate caused cell death, micronuclei formation, chromosomal aberrations and sister chromatid exchange in CHO cells (25). It caused an increase in dicentric chromosomes (26), oxidative damage in the presence of H_2O_2 (27) and micronuclei formation (28) in human osteoblast cells. Uranyl chloride increased sister chromatid exchange and transformed human osteoblast cells (29) and induced apoptosis in mouse J774 macrophages (30). The current work has shown that UA will induce mutations in the DNA repair-deficient CHO EM9 line. This is the first report of mutations produced by direct exposure to UA, but it is also consistent with previous work showing mutations in the Ames Salmonella reversion assay with exposure to urine from rats with imbedded DU pellets (31) and a slight increase in *hprt* mutations in lymphocytes of Gulf war veterans with imbedded DU shrapnel (9).

UA appears to be a weak mutagen compared to other chemicals that have been tested in CHO cells by this assay, producing an average induced mutant frequency (AIMF) of $31/10^6$ surviving cells in the EM9 line (Table I). Other agents induce much higher AIMF in repair-proficient cells. Methamphetamine showed an AIMF of $13/10^6$ in the CHO K1 line (32). The dietary supplement chromium picolinate produced an AIMF of $58/10^6$ surviving cells in the AA8 line (14). ^{60}Co γ rays produced $116/10^6$ surviving cells in the AA8 line (33). Stronger responses were observed with the alkylating agents ethyl methanesulfonate (20 mM, 1 h) and *N*-ethyl-*N*-nitrosourea (1.5 mM, 1 h), both exposures producing ∼1000 mutants/10^6 surviving cells (34). The alkylating agent *N*-*n*-butyl-*N*-nitrosourea (2 mM, 1 h) was less mutagenic, producing ∼410 mutants/10^6 survivors (35). However, the low mutant frequency of UA at the *hprt* locus measured in the current study may underrepresent UA mutagenicity since this assay would not detect mutants harboring multilocus mutations. Large multilocus mutations may inactivate essential genes neighboring *hprt*, causing lethality in those mutant cells since there is no homologous X chromosome to supply the essential gene. This interpretation is consistent with the observation that multiexon deletions were a dominant mutation in UA-exposed CHO EM9 cells (36).

The purpose of the current study was to begin to acquire mechanistic information. Therefore, the exposure levels in these experiments are higher than those found in drinking water. There is also evidence that cell lines may differ in their sensitivity to uranyl ion. The CI_{50} of uranyl nitrate and UA in kidney cells was found to be 500–650 μM for 24 h exposures in rat, human and porcine kidney proximal tubule cell lines (37,38). However, results from a short-term MTT assay cannot be directly compared to the colony formation assay in the current study. The human osteoblast (HOS) line appears to be more sensitive to uranyl ion than the CHO line, with a 24 h exposure to 100 μM uranyl chloride producing a 0.1 survival fraction by clonogenic assay (29) versus 87% survival for this dose of UA in the current study. However, this interpretation must be tempered by the possibility that the $^{238}U/^{235}U$ isotopic ratios could vary with the different forms of depleted uranium used in these studies, with more ^{235}U causing more radiological toxicity. Also different relative concentrations of components in the cell culture medium for these different lines, for example carbonate or phosphate, could influence uranyl speciation, affecting uptake or bioactivity.

Uranium has generally been considered to be DNA damaging through its radioactivity, specifically through release of alpha and beta particles during its radioactive decay; however, chemical mechanisms may also exist. Combinations of depleted uranium as UA and ascorbate were found to produce DNA strand breaks in plasmid DNA that were greater than those for either UA or ascorbate alone, and were observed in the absence of ascorbate-induced reduction of uranyl ion (10), which suggested a direct chemical mechanism for uranium, ascorbate and DNA interactions because the half-lives for decay of uranium isotopes would not be changed by the addition of ascorbate.

Heavy metals in general have been considered to cause DNA damage through indirect mechanisms of free radical generation and oxidative stress. For example nickel, copper, iron and chromium are believed to either undergo electron

transfer reactions with biological reducing agents or have their redox potentials altered by chelation with biomolecules, producing a metal complex that reacts with O_2 or H_2O_2 to generate HO· or other reactive oxygen species (39–42). However, data from the current study and our previous work (10) suggest that, at least in the absence of added hydrogen peroxide, direct uranium(VI)–DNA interactions are more important than free radical mechanisms. If free radical generation were a major pathway under the current conditions, then it would have been expected that oxidative damage would have been detected by the comet assay (Figure 3).

Another mechanism by which metals damage DNA is by a direct covalent interaction. This pathway is known to be important for chromium (43), and it may occur for uranium as well. Uranium has been known to interact with DNA *in vitro* (44–46); however, to our knowledge this is the first report of U–DNA adducts recovered from cultured cells. The current experiments found that uranium covalently bonded to DNA; however, at this time data cannot distinguish between simple uranium–DNA adducts and uranium-containing DNA–protein crosslinks or uranium-containing DNA–DNA crosslinks. The observation of modest differences in adduct levels between these two cell lines is consistent with the interpretation that the CHO EM9 line is depleted in XRCC1-ligase complex, and is therefore less sensitive to crosslinks than strand breaks. Current work is in progress to measure U–DNA adducts in crosslink-sensitive lines.

The current experiments did show evidence of DNA strand breaks in CHO cells exposed to UA (Figure 3). This is consistent with other studies reporting chromosomal aberrations in CHO cells exposed to uranyl nitrate (25) and in mouse germ cells exposed to enriched uranyl fluoride (47). However, because strand breaks were not the only DNA lesion observed, it is not yet clear whether the strand breaks detected in the comet assay were caused by direct action of uranium on DNA, for example DNA hydrolysis catalyzed by uranium coordinating to the DNA phosphate backbone (10), or were indirect intermediates of DNA excision repair. Nevertheless, the lack of oxidative damage in the comet assay coupled with the presence of U–DNA adducts suggests that uranium is acting through a chemical rather than a radiological mechanism.

In conclusion, depleted uranium as UA was found to be genotoxic and mutagenic in CHO cells. The presence of U–DNA adducts lends further support to the hypothesis that uranium is chemically genotoxic. This possibility of direct U–DNA interaction should be considered when extrapolating potential risks for people exposed to uranium in the absence of measurable radioactivity, for example in soil and drinking water, and in DU-containing shrapnel.

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References

1. Churchill,W. (1996) *From a Native Son: Selected Essays in Indigenism, 1985–1995*. South End Press, Cambridge, MA, p. 27.
2. Durakovic,A. (2003) Undiagnosed illnesses and radioactive warfare. *Croatian Med. J.*, **44**, 520–532.
3. Mulloy,K.B., James,D.S., Mohs,K. and Kornfeld,M. (2001) Lung cancer in a nonsmoking underground uranium miner. *Environ. Health Perspect.*, **1009**, 305–309.
4. Gilliand,F.D., Hunt,W.C., Pardilla,M. and Key,C.R. (2000) Uranium mining and lung cancer among Navajo men in New Mexico and Arizona, 1969–1993. *J. Occup. Environ. Med.*, **42**, 278–283.
5. Chen,J., Meyerhof,D.P. and Tracy,B.L. (2004) Model results of kidney burdens from uranium intakes. *Health Phys.*, **86**, 3–11.
6. Pinney,S.M., Freyberg,R.W., Levine,G.E., Brannen,D.E., Mark,L.S., Nasuta,J.M., Tebbe,C.D., Buckholz,J.M. and Wones,R. (2003) Health effects in community residents near a uranium plant at Fernald, Ohio, USA. *Int. J. Occup. Med. Environ. Health*, **16**, 139–153.
7. Shields,L.M., Wiese,W.H., Skipper,B.J., Charley,P. and Benally,L. (1992) Navajo birth outcomes in the Shiprock uranium mining area. *Health Phys.*, **63**, 542–551.
8. Au,W.W., Lane,R.G., Legator,M.S., Whorton,E.B., Wilkinson,G.S. and Gabehart,G.J. (1995) Biomarker monitoring of a population residing near uranium mining activities. *Environ. Health Perspect*, **103**, 466–470.
9. McDiarmid,M.A., Engelhardt,S., Oliver,M. *et al.* (2004) Health effects of depleted uranium on exposed Gulf War veterans: a 10-year follow-up. *J. Toxicol. Environ. Health A*, **67**, 277–296.
10. Yazzie,M., Gamble,S.L., Civitello,E.R. and Stearns,D.M. (2003) Uranyl acetate causes DNA single strand breaks *in vitro* in the presence of ascorbate (vitamin C). *Chem. Res. Toxicol.*, **16**, 524–530.
11. Caldecott,K.W., Tucker,J.D., Stanker,L.H. and Thompson,L.H. (1995) Characterization of the XRCCa-DNA ligase III complex *in vitro* and its absence from mutant hamster cells. *Nucleic Acid Res.*, **23**, 4836–4843.
12. Thompson,L.H. and West,M.G. (2000) XRCC1 keeps DNA from getting stranded. *Mutat. Res.*, **459**, 1–18.
13. O'Neill,J.P. and Hsie,A.W. (1979) The CHO/HGPRT mutation assay: experimental procedures. In Hsie,A.W., O'Neill,J.P. and McElheny,V.K. (eds), *Banbury Report 2. Mammalian Cell Mutagenesis: The Maturation of Test Systems*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 55–69.
14. Stearns,D.M., Silveira,S.M., Wolf,K.K. and Luke,A.M. (2002) Chromium(III) tris(picolinate) is mutagenic at the hypoxanthine (guanine) phosphoribosyltransferase locus in Chinese hamster ovary cells. *Mutat. Res.*, **513**, 135–142.
15. Tice,R.R., Agurell,E., Anderson,D. *et al.* (2000) Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.*, **35**, 206–221.
16. Singh,N.P. (2000) Microgels for estimation of DNA strand breaks, DNA-protein crosslinks and apoptosis. *Mutat. Res.*, **455**, 111–127.
17. Cantoni,O., Murray,D. and Meyn,R.E. (1987) Induction and repair of DNA single-strand breaks in EM9 mutant CHO cells treated with hydrogen peroxide. *Chem. Biol. Interact.*, **63**, 29–38.
18. Martin,I.V. and MacNeill,S.A. (2002) ATP-dependent DNA ligases. *Genome Biol.*, **3**, 3005.1–3005.7.
19. Leppard,J.B., Dong,Z., Mackey,Z.B. and Tomkinson,A.E. (2003) Physical and functional interaction between DNA ligase IIIalpha and poly(ADP-Ribose) polymerase 1 in DNA single-strand break repair. *Mol. Cell Biol.*, **23**, 5919–5927.
20. Pfuhler,S. and Wolf,H.U. (1996) Detection of DNA-crosslinking agents with the alkaline comet assay. *Environ. Mol. Mutagen.*, **27**, 196–201.
21. Christie,N.T., Cantoni,O., Evans,R.M., Meyn,R.E. and Costa,M. (1984) Use of mammalian DNA repair-deficient mutants to assess the effects of toxic metal compounds on DNA. *Biochem. Pharmacol.*, **33**, 1661–1670.
22. Churchill,M.E., Peak,J.G. and Peak,M.J. (1991) Correlation between cell survival and DNA single-strand break repair proficiency in the Chinese hamster ovary cell lines AA8 and EM9 irradiated with 365-nm ultraviolet-A radiation. *Photochem. Photobiol.*, **53**, 229–236.
23. Churchill,M.E., Peak,J.G. and Peak,M.J. (1991) Repair of near-visible- and blue-light-induced DNA single-strand breaks by the CHO cell lines AA8 and EM9. *Photochem. Photobiol.*, **54**, 639–644.
24. Thompson,L.H., Brookman,K.W., Dillehay,L.E., Carrano,A.V., Mazrimas,J.A., Mooney,C.L. and Minkler,J.L. (1982) A CHO-cell strain having hypersensitivity to mutagens, a defect in DNA strand-break repair, and an extraordinary baseline frequency of sister-chromatid exchange. *Mutat. Res.*, **95**, 427–440.
25. Lin,R.H., Wu,L.J., Ching,H.L. and Lin-Shiau,S.Y. (1993) Cytogenic toxicity of uranyl nitrate in Chinese hamster ovary cells. *Mutat. Res.*, **319**, 197–203.
26. Miller,A.C., Xu,J., Stewart,M., Brooks,K., Hodge,S., Shi,L., Page,N. and McClain,D. (2002a) Observation of radiation-specific damage in human cells exposed to depleted uranium: dicentric frequency and neoplastic transformation as endpoints. *Radiat. Prot. Dosim.*, **99**, 275–278.

27. Miller,A.C., Stewart,M., Brooks,K., Shi,L. and Page,N. (2002b) Depleted uranium-catalyzed oxidative DNA damage: absence of significant alpha particle decay. *J. Inorg. Biochem.*, **91**, 246–252.
28. Miller,A.C., Brooks,K., Stewart,M., Anderson,B., Shi,L., McClain,D. and Page,N. (2003) Genomic instability in human osteoblast cells after exposure to depleted uranium: delayed lethality and micronuclei formation. *J. Environ. Radioact.*, **64**, 247–259.
29. Miller,A.C., Blakely,W.F., Livengood,D. *et al.* (1998a) Transformation of human osteoblast cells to the tumorigenic phenotype by depleted uranium-uranyl chloride. *Environ. Health Perspect.*, **106**, 465–471.
30. Kalinich,J.F., Ramakrishnan,N., Villa,V. and McClain,D.E. (2002) Depleted uranium-uranyl chloride induces apoptosis in mouse J774 macrophages. *Toxicology*, **179**, 105–114.
31. Miller,A.C., Fuciarelli,A.F., Jackson,W.E., Ejnik,E.J., Emond,C., Strocko,S., Hogan,J., Page,N. and Pellmar,T. (1998b) Urinary and serum mutagenicity studies with rats implanted with depleted uranium or tantalum pellets. *Mutagenesis*, **13**, 643–648.
32. Li,J.-H., Hu,H.-C., Chen,W.-B. and Kin,S.-K. (2003) Genetic toxicity of methamphetamine *in vitro* and in human abusers. *Environ. Mol. Mutagen.*, **42**, 233–242.
33. Diamond,A.M., Dale,P., Murray,J.L. and Grdina,D.J. (1996) The inhibition of radiation-induced mutagenesis by the combined effects of selenium and the aminothiol WR-1065. *Mutat. Res.*, **356**, 147–154.
34. Op het Veld,C.W., van Hees-Stuivenberg,S., van Zeeland,A.A. and Jansen,J.G. (1997) Effect of nucleotide excision repair on *hprt* gene mutations in rodent cells exposed to DNA ethylating agents. *Mutagenesis*, **12**, 417–424.
35. Bol,S.A., van Steeg,H., van Oostrom,C.T., Tates,A.D., Vrieling,H., de Groot,A.J., Mullenders,L.H., van Zeeland,A.A. and Jansen,J.G. (1999) Nucleotide excision repair modulates the cytotoxic and mutagenic effects of N-n-butyl-N-nitrosourea in cultured mammalian cells as well as in mouse splenocytes *in vivo*. *Mutagenesis*, **14**, 317–322.
36. Coryell,V.H. and Stearns,D.M. (2005) Molecular analysis of *hprt* mutations generated bin Chinese hamster ovary EM9 cells by uranyl acetate, by hydrogen peroxide and spontaneously. *Molec. Carcinogen.*, in press.
37. Carrière,M., Avoscan,L., Collins,R., Carrot,F., Khodja,H., Ansoborlo,E. and Gouget,B. (2004) Influence of uranium speciation on normal rat kidney (NRK-52E) proximal cell cytotoxicity. *Chem. Res. Toxicol.*, **17**, 446–452.
38. Mirto,H., Barrouillet,M.P., Henge-Napoli,M.H., Ansoborlo,E., Fournier,M. and Cambar,J. (1999) Influence of uranium(VI) speciation for the evaluation of *in vitro* uranium cytotoxicity on LLC-PK1 cells. *Hum. Exp. Toxicol.*, **18**, 180–187.
39. Kawanishi,S., Hiraku,Y., Murata,M. and Oikawa,S. (2002) The role of metals in site-specific DNA damage with reference to carcinogenesis. *Free Radic. Biol. Med.*, **32**, 822–832.
40. Schumann,K., Classen,H.G., Dieter,H.H., Konig,J., Multhaup,G., Rukgauer,M., Summer,K.H., Bernhardt,J. and Biesalski,H.K. (2002) Hohenheim consensus workshop: copper. *Eur. J. Clin. Nutr.*, **56**, 469–483.
41. Toyokuni,S. (1996) Iron-induced carcinogenesis: the role of redox regulation. *Free Radic. Biol. Med.*, **20**, 553–566.
42. Shi,X., Chiu,A., Chen,C.T., Halliwell,B., Castranova,V. and Vallyathan,V. (1999) Reduction of chromium(VI) and its relationship to carcinogenesis. *J. Toxicol. Environ. Health B Crit. Rev.*, **2**, 87–104.
43. O'Brien,T.J., Ceryak,S. and Patierno,S.R. (2003) Complexities of chromium carcinogenesis: role of cellular response, repair and recovery mechanisms. *Mutat. Res.*, **533**, 3–36.
44. Zobel,C.R. and Beer,M. (1961) Electron stains. I. Chemical studies on the interaction of DNA with uranyl salts. *J. Biophys. Biochem. Cytol.*, **10**, 335–346.
45. Drynov,I.D., Poletaev,A.I., Kharitonenkov,I.G. and Klimenko,S.M. (1974) The interaction of uranyl acetate with DNA. *Mol. Biol.*, **8**, 27–33.
46. Jeppesen,C. and Nielsen,P.E. (1989) Uranyl mediated photofootprinting reveals strong *E. coli* RNA polymerase—DNA backbone contacts in the +10 region of the DeoP1 promoter open complex. *Nucleic Acids Res.*, **17**, 4947–4956.
47. Hu,Q.Y. and Zhu,S.P. (1990) Induction of chromosomal aberrations in male mouse germ cells by uranyl fluoride containing enriched uranium. *Mut. Res.*, **244**, 209–214.

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Drinking Water with Uranium below the U.S. EPA Water Standard Causes Estrogen Receptor–Dependent Responses in Female Mice

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BACKGROUND: The deleterious impact of uranium on human health has been linked to its radioactive and heavy metal–chemical properties. Decades of research has defined the causal relationship between uranium mining/milling and onset of kidney and respiratory diseases 25 years later.

OBJECTIVE: We investigated the hypothesis that uranium, similar to other heavy metals such as cadmium, acts like estrogen.

METHODS: In several experiments, we exposed intact, ovariectomized, or pregnant mice to depleted uranium in drinking water [ranging from 0.5 µg/L (0.001 µM) to 28 mg/L (120 µM).

RESULTS: Mice that drank uranium-containing water exhibited estrogenic responses including selective reduction of primary follicles, increased uterine weight, greater uterine luminal epithelial cell height, accelerated vaginal opening, and persistent presence of cornified vaginal cells. Coincident treatment with the antiestrogen ICI 182,780 blocked these responses to uranium or the synthetic estrogen diethylstilbestrol. In addition, mouse dams that drank uranium-containing water delivered grossly normal pups, but they had significantly fewer primordial follicles than pups whose dams drank control tap water.

CONCLUSIONS: Because of the decades of uranium mining/milling in the Colorado plateau in the Four Corners region of the American Southwest, the uranium concentration and the route of exposure used in these studies are environmentally relevant. Our data support the conclusion that uranium is an endocrine-disrupting chemical and populations exposed to environmental uranium should be followed for increased risk of fertility problems and reproductive cancers.

KEY WORDS: depleted uranium, endocrine disruption, estrogen, estrogen receptor, female reproduction, heavy metal, Navajo reservation. *Environ Health Perspect* 115:1711–1716 (2007). doi:10.1289/ehp.9910 available via *http://dx.doi.org/* [Online 14 September 2007]

Uranium, the heaviest naturally occurring element, is valued for its radioactive properties. Development of nuclear weapons in the 1940s fueled the U.S. government's desire to become independent of foreign sources of U (Ball 1993; Moure-Eraso 1999; Panikkar and Brugge 2007). The U "boom" in the southwestern United States lasted from the early 1950s until the market collapsed in 1971, when the U.S. government ceased being the sole purchaser of U ore (Brugge and Goble 2002).

The majority of U mining/milling occurred in the Four Corners region of the United States where the Navajo Reservation is located. The Navajo Abandoned Mine Lands (AML) agency reclaims abandoned uranium mines (AUMs) under the authority and with funding from the Surface Mining Control and Reclamation Act of 1977 (Office of Surface Mining 1977). The Navajo AML agency has estimated that there are approximately 1,300 AUMs throughout the 27,000 square miles of the Navajo Nation (U.S. EPA 2004). About 50% of AUMs have been reclaimed [U.S. Environmental Protection Agency (EPA) 2004]. Unremediated AUMs enabled U to disperse into air, soil, water, and the food chain (Brugge and Goble 2002). A present-day example of unregulated U mining/milling is the Atlas Corporation Moab Uranium Mill Tailing (Moab, UT). Nearly 10,000 gallons of U-contaminated water seeps into the Colorado River daily (Oak Ridge National Laboratory 1998), and the adjacent surface water concentration of uranium is > 5 mg/L (Department of Energy 2005).

The largest American Indian reservation in the United States is the Navajo Nation, which is divided into 110 political units called Chapters. Within 33 Chapters, the U.S. EPA surveyed 226 water sources. Of these, 90 water sources were contaminated with U above the U.S. EPA safe drinking water level of 30 µg/L (0.126 µM). The U levels found in contaminated water sources ranged from 33.3 to 1,131 µg/L, with the highest concentration being 38 times the safe drinking water level (U.S. EPA 2004). The surveyed water sources were stock tanks, wells, and springs. Chapter officials identified the water sources as providing drinking water for residents without running water (U.S. EPA 2004). According to the 2000 U.S. census (2006), > 175,000 people live on the Navajo Reservation. At least half of these residents haul water from the nearest water source for household use (i.e., drinking water, cooking, and clothes laundering), making it a certainty that many Navajo Nation residents are exposed to unsafe levels of U.

The toxicity of U is due to its radioactive and chemical properties (Brugge et al. 2005; Taylor and Taylor 1997). U inhalation and/or ingestion leads to malignant and nonmalignant respiratory diseases, stomach and kidney cancer, kidney failure, and leukemia (Brugge et al. 2005; Roscoe et al. 1995). U's effect on the reproductive system was examined in early studies with rats fed high doses of 2% uranyl nitrate (UN). U exposure caused significant weight loss in dams, fewer litters, and fewer pups per litter (Maynard and Hodge 1949). When female rats were returned to chow diet without UN, they regained the lost body weight, but a reduction in the number of litters and pups per litter persisted, suggesting that the ovaries had been permanently damaged (Maynard and Hodge 1949). Female mice treated with uranyl acetate by gavage through gestation, parturition, and nursing had an increased number of dead young per litter (Paternain et al. 1989). It is likely that the high doses of U in these studies led to reproductive toxicity (Domingo 2001; Hindin et al. 2005).

Heavy metals exhibit estrogenic properties (Dyer 2007). Several heavy metals stimulate proliferation of MCF-7 human breast cancer cells (Brama et al. 2007; Choe et al. 2003; Martin et al. 2003; Martinez-Campa et al. 2006). Cadmium interacts with estrogen receptor-α (ER-α) (Brama et al. 2007; Martin et al. 2003) and binds to the ligand-binding domain of ER-α in cultured cells (Stoica et al. 2000). Cd stimulates estrogenic responses *in vivo* (Alonso-Gonzalez et al. 2007; Johnson et al. 2003). Ovariectomized rats injected with Cd had increased uterine

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weight, accelerated mammary gland growth/development, and accelerated vaginal opening (VO) (Johnson et al. 2003). Cd-induced estrogen-like responses were prevented by the antiestrogen ICI 182,780. Cd inhibits transcriptional activity of estradiol-activated rainbow trout ER in recombinant yeast (Guével et al. 2000). Cd treatment stimulates breast cancer cell proliferation by activating ER-α–dependent Akt (protein kinase B), Erk1/2 (extracellular signal-regulated kinase), and platelet-derived growth factor receptor-α (Brama et al. 2007). Although these studies demonstrate the estrogen activity of Cd, it should be noted that Silva et al. (2006) reported that Cd lacks estrogenic activity in the yeast estrogen screen assay, MCF-7 cell proliferation, or the E-SCREEN assay, and also failed to induce Src, Erk1, and Erk2 phosphorylation. In the present study we tested whether depleted U added to drinking water caused responses in the female mouse reproductive tract like those caused by the potent synthetic estrogen diethylstilbestrol (DES).

Materials and Methods

Animals. We performed U exposure in intact female mice using 28-day-old immature B6C3F$_1$ mice (Harlan, Indianapolis, IN). For *in utero* U exposure experiments, we used 48-day-old male and female B6C3F$_1$ mice (Harlan). We used ovariectomized 28-day-old C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) for the prepubertal U and DES exposure experiments. Mice were housed with a 12:12 hr light/dark cycle and received water and food *ad libitum.* Control tap water tested for U using kinetic phosphorescence analysis, as described by Hedaya et al. (1997), was below the limit of detection (< 2 μg/L or < 8 pM). All protocols were approved by the University of Arizona or Northern Arizona University Institutional Animal Care and Use Committees. All mice were treated humanely with regard for alleviation of suffering in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Research, 1996).

Treatments. Animals were treated with UN hexahydrate (depleted U) (Sigma Chemical Co., St. Louis, MO) in drinking water.

Study 1: Impact of U exposure on ovarian follicle populations. *Experiment 1.1: U exposure in immature mice.* Mice were exposed to UN in their drinking water at milligram per liter doses based on a study using rats (Gilman et al. 1998). Immature 28-day-old B6C3F$_1$ mice drank water containing UN at 0.5, 2.5, 12.5, and 60.0 mg/L (1, 5, 25, and 120 μM, respectively; n = 9–10 mice per group). After 30 days, we analyzed ovaries for changes in follicle populations.

Experiment 1.2: Gestational and in utero *U exposure in dams and female pups.* For *in utero* exposure, mice were given water containing UN at 0.5, 2.5, 12.5, or 60 μg/L (0.001, 0.005, 0.025, or 0.120 μM U, respectively) for 30 days prior to breeding. U dose was reduced a thousandfold to micrograms per liter to correspond to environmentally relevant concentrations. Mice were paired for breeding, and males were removed when females had vaginal plugs. Females continued to drink U-containing water at the above doses through gestation. On the day of birth, dams (n = 5 mice per treatment group) and female pups (n = 7–9 pups per treatment group) were euthanized and the ovaries collected for histology.

Study 2: Impact of U exposure on the female reproductive tract in the absence of endogenous estrogen. *Experiment 2.1: U exposure in ovariectomized mice.* For this study we used C57Bl/6J mice because of strain sensitivity to estrogen in the uterotrophic assay (Ashby et al. 2003). We also anticipated the use of genetically manipulated mice (e.g., ER-α knockout mice) on this genetic background (Lubahn et al. 1993). C57Bl/6J mice were ovariectomized at 28 days of age to remove the endogenous source of estrogen before VO. Seven days postsurgery, ovariectomized and intact mice were given tap water or water containing 0.19 μM DES or 0.06, 0.12, 1.20, or 12.00 μM U for 30 days (n = 5–6 mice per treatment group).

Experiment 2.2: Other estrogen-like effects of UN and dependence on ER activation. Mice ovariectomized at 28 days of age were exposed to drinking water containing U or DES at the aforementioned concentrations for 10 days beginning at 50 days of age. Some mice (n = 6–7 mice per group) concurrently received daily intraperitoneal (i.p.) injections of either sesame oil vehicle or 500 μg/kg ICI 182,780 (Tocris Coolson Ltd., Avonmouth, UK). Mice were examined daily at the same time for VO and cytology.

Tissue collection and histology. After exposure to DES or U, mice were euthanized and organs were collected for necropsy. Uteri were removed by dissecting inferior to the Fallopian tubes and superior to the vagina. Wet weights of ovary, uterus, kidney, liver, and spleen were normalized to total body weight. Uterine tissues were fixed in Bouin's solution, embedded in paraffin, and serially sectioned every 9 μm; every 10th section was mounted on slides. Tissue sections were deparaffinized in Citrasolve (Sigma Chemical Co.) and dehydrated in a series of ethanol baths. We used a Zeiss 435 VP scanning electron microscope and LEO32 V02.01 software (Carl Zeiss SMT Inc., Peabody, MA) to measure the height of uterine luminal epithelial cells. Forty measurements were randomly collected from each individual uterus.

Ovaries were trimmed of adhering tissue and fat and then fixed in Bouin's solution. They were transferred to 70% ethanol, embedded in paraffin, serially sectioned (5 μm), mounted, and stained with hematoxylin and eosin. Nuclei of oogonia and primordial, small primary, large primary, secondary or growing, and healthy antral and atretic follicles were identified and counted in adult ovary every 20th section, and in pup ovary every 12th section (Mayer et al. 2004).

Statistical analyses. Oogonia and follicle numbers were determined in ovaries from individual mice and averaged. The means in control versus exposed mice were analyzed for significant differences by one-way analysis of

Table 1. Effects of UN exposure on specific ovarian follicle populations (follicle counts per ovary; mean ± SE) in B63CF$_1$ mice exposed to UN in drinking water for 30 days.

Follicle type	Control (U < 0.002 mg/L)	UN (mg/L) 0.5	2.5	12.5	60.0
Primordial	65.55 ± 7.05	53.80 ± 8.26	37.88 ± 7.01	57.60 ± 13.29	61.60 ± 12.76
Small primary	26.22 ± 2.50	19.40 ± 3.03	18.56 ± 2.94	32.00 ± 3.51	21.78 ± 2.81
Large primary	12.66 ± 0.69	6.50 ± 1.17*	7.44 ± 1.27*	12.00 ± 1.51	9.11 ± 0.65
Secondary or growing	26.44 ± 1.08	24.20 ± 2.09	21.22 ± 1.85	33.30 ± 1.92*	26.78 ± 0.81
Healthy antral	31.22 ± 2.56	31.00 ± 3.49	28.22 ± 3.71	29.00 ± 2.39	23.11 ± 2.78
Atretic antral	17.22 ± 1.37	15.50 ± 2.37	11.44 ± 1.70	16.00 ± 3.26	12.53 ± 1.37

n = 6 per group.
*Significantly different from control ($p < 0.05$, Tukey-Kramer post hoc test).

Table 2. Effects of UN exposure on body weight and tissue weight in B63CF$_1$ mice exposed to UN in drinking water for 30 days.

Treatment	Body weight	Ovary	Uterus	Liver	Adrenal	Kidney	Spleen
Control (< 2 μg/L U)	100.0	100.0	100.0	100.0	100.0	100.0	100.0
U (mg/L)							
0.5	101.2	77.5	97.1	94.2	95.5	96.0	104.0
2.5	100.4	72.5	81.8	94.4	88.4	91.7*	89.9
12.5	104.1	73.9	115.9	99.2	120.8	100.9	103.6
60.0	104.6	62.4	127.8	110.6	108.5	94.2*	109.8

Tissue weights are expressed as a percent of control values normalized to total body weight.
*Significantly different from control ($p < 0.05$).

variance (ANOVA) with significance set at $p < 0.05$. We used Tukey-Kramer post hoc tests where appropriate. For mice exposed for 10 and 30 days, organ weights were determined for each individual within each experiment and averaged for each exposure group. In the 30-day–exposure group, uterine luminal epithelial cell height measurements were collected from individual mice and averaged for each exposure group. Additionally, in the 10-day–exposure group, VO was determined for each individual and averaged for the exposure group. The means for control versus exposed mice for organ weights, uterine epithelial cell height, and VO were analyzed for significant differences by one-way ANOVA with significance set at $p < 0.05$. We used Dunnet's post hoc test where appropriate. The means of uterine weights in controls or in mice exposed to ICI 182,780, U, or DES were analyzed by two-way ANOVA with significance set at $p < 0.05$. Persistent presence of cornified vaginal cells was determined for each individual mouse in the 10-day–exposure group. Presence and absence of cornified cells was analyzed by chi-square test with significance set at $p < 0.05$. Statistical significance of persistent presence of cornified cells was analyzed by Fisher's exact test with significance set at $p < 0.05$.

Results

Study 1: Impact of U exposure on ovarian follicle populations. **Experiment 1.1: U exposure in immature mice.** Experiment 1.1: showed that U targets early stage ovarian follicles. As shown in Table 1, there were significantly fewer large primary follicles at 0.5 and 2.5 mg/L UN and significantly more secondary or growing follicles at 12.5 mg/L UN. However, we found no significant increase in the number of atretic follicles or decrease in healthy follicles. Because UN exposure caused a selective change in ovarian follicle populations and because there were more growing follicles at 12.5 mg/L UN, the changes could not be caused by heavy metal toxicity.

This experiment also showed that U does not lead to overt organ toxicity. We found no gross anomalies in any major organs, and body weight did not significantly change with UN exposure at any concentration. As shown in Table 2, kidney weight was significantly reduced at doses of 2.5 and 60.0 mg/L UN, but this was not surprising given the nephrotoxicity of U (Brugge et al. 2005; Taylor and Taylor 1997). These data support the conclusion that there was no systemic UN-mediated toxicity.

We found an interesting, but not statistically significant, trend of increased uterine weight at 12.5 and 60.0 mg/L UN (Table 2). We did not determine estrous cycle stage in mice at sacrifice, thus uterine weights could not be grouped relative to stage.

Experiment 1.2: Gestational and *in utero* U exposure in dams and female pups. Experiment 1.2 showed that *in utero* uranium exposure reduces pup ovary primordial follicles. As shown in Figure 1A, mice exposed to UN for 30 days before mating and through gestation had a significant reduction of small primary follicles at UN concentrations of 0.005, 0.025, and 0.120 µM compared with control mice. All other follicle populations, including primordial, secondary/growing, healthy, and atretic, were unchanged (data not shown). Neonatal mouse ovaries have only oogonia and primordial follicles. We found no difference in the number of pup ovary oogonia among control and UN exposure groups (data not shown). Primordial follicle numbers were reduced in ovaries of pups whose dams consumed water with 0.001- or 0.120-µM UN, compared with primordial follicles in pup ovaries from dams drinking control tap water (Figure 1B).

Study 2: Impact of U exposure on the female reproductive tract in the absence of endogenous estrogen. **Experiment 2.1: U exposure in ovariectomized mice.** Experiment 2.1 showed that UN exposure induces estrogen-like changes in uterine morphology and histology. Mice exposed to UN or DES had significantly increased uterine weight at 0.120 µM U and 0.19 µM DES, 3.6 and 3.8 times greater, respectively, compared with mice drinking control tap water (Figure 2A). We normalized uterine weights to body weights, which were unchanged across treatment groups. Uterine weights were not increased in ovary-intact, age-matched mice that drank U-containing water (data not shown).

Experiment 2.2: Other estrogen-like effects of UN and their mediation through ER activation. Experiment 2.2 showed that UN-mediated estrogen-like actions are blocked by concomitant exposure to an ER antagonist. To determine if the U-mediated uterotrophic response was dependent on ER activation, ovariectomized mice drinking UN-containing water were injected daily with the antiestrogen ICI 182,780. In a pilot experiment, we determined that 10 days of exposure to UN in drinking water caused a significant increase in uterine weight compared with mice drinking tap water (data not shown). Ten days of concomitant ICI 182,780 treatment blocked both UN- and DES-mediated increases in uterine weights (Figure 2B): 0.06 µM U alone, 1,070 ± 386 mg/kg total bw; 0.06 µM U plus ICI 182,780, 220 ± 28.1 mg/kg total bw; 0.19 µM DES alone, 1,530 ± 282 mg/kg total bw; 0.19 µM DES plus ICI 182,780, 252 ± 24.7 mg/kg total bw. Uterine weights of control mice were

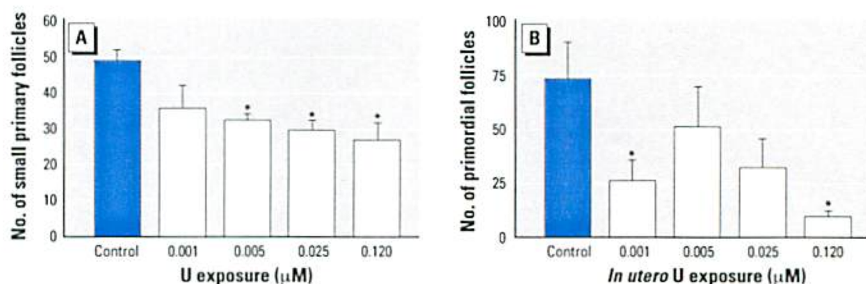

Figure 1. Effects of UN at 0.5, 2.5, 12.5, or 60 µg/L (0.001, 0.005, 0.025, or 0.120 µM U, respectively) on dam follicle populations and *in utero* exposed pup ovary primordial follicles. B6C3F$_1$ dams were exposed to control tap water or U in drinking water for 30 days before mating and through gestation. Ovaries from dams (*A*) and pups (*B*) were removed on the day of birth. Values shown are mean ± SE ($n = 7–11$).
*Significantly different compared with controls ($p < 0.05$, ANOVA).

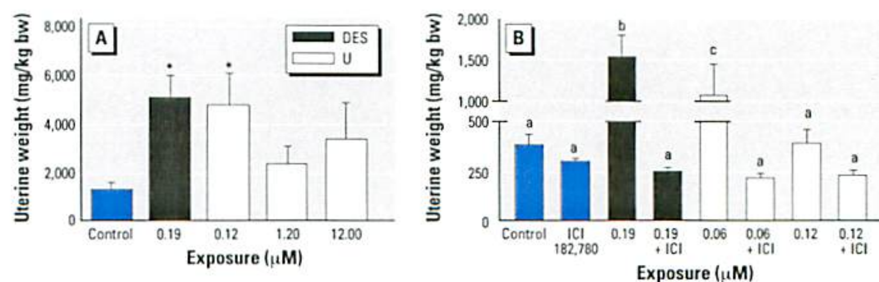

Figure 2. Effect of UN or DES alone and in combination with ICI 182,780 on uterine weight in ovariectomized C57Bl/6J mice. (*A*) Uteri were removed after 30 days of exposure, and wet weights were recorded and normalized to body weight; values shown are mean ± SE ($n = 5–6$). (*B*) Uteri were removed after 10 days of exposure, and wet weights were recorded and normalized to body weight; values shown are mean ± SE ($n = 6–7$). Different letters (a, b, c) indicate significant differences among exposure groups ($p < 0.005$).
*Significantly different compared with other exposure groups ($p < 0.001$).

not significantly different from controls treated with ICI 182,780 (Figure 2B).

One aspect of the uterotrophic response to estrogen is proliferation of the epithelial cell lining of the uterus (Kang et al. 1975; O'Brien et al. 2006). Uterine epithelial cell height was significantly greater in mice drinking water containing U or DES for 30 days (Figure 3A); 0.120 µM U, 31.01 ± 1.89 µm; 1.20 µM U: 23.79 ± 0.93 µm; 0.19 µM DES, 40.2 ± 1.85 µm; controls, 15.24 ± 0.77 µm. Figures 3B (control), 3C (0.19 µM DES), and 3D (0.12 µM U) show scanning electron micrographs illustrating changes in uterine luminal epithelial cell height. Arrows in in Figure 3C and 3D indicate pseudostratified columnar morphology typical of proliferating epithelial cells due to DES or UN exposure, respectively.

Effects of U on VO and vaginal cell cornification. Estrogen and endocrine-disrupting chemicals (EDCs) accelerate VO in mice (Markey et al. 2001). Ovariectomized mice exposed to 0.12 µM UN or 0.19 µM DES exhibited significantly accelerated VO (both at 52.5 days), compared with control mice (54 days) (Figure 4A). UN- or DES-mediated acceleration of puberty onset, as indicated by day of VO, was prevented by concomitant treatment with the antiestrogen ICI 182,780 (Figure 4A).

Another indication of estrogenic influence on the female reproductive tract is the persistent presence of cornified cells in vaginal smears (Gordon et al. 1986). As shown in Figure 4B, mice exposed to 0.06 µM UN (4 mice) or 0.12 µM UN (5 mice), or 0.19 µM DES (6 mice) had persistent presence of cornified vaginal cells compared with control mice (0 mice). Coincident treatment with ICI 182,780 prevented the presence of cornified vaginal cells (0.06 µM UN, 0 mice; 0.12 µM UN, 0 mice; 0.19 µM DES, 1 mouse).

Discussion

The major contribution of the present study is the discovery that U, similar to other heavy metals, has estrogenic activity (Alonso-Gonzalez et al. 2007; Brama et al. 2007; Choe et al. 2003; Dyer 2007; Johnson et al. 2003; Martin et al. 2003; Martinez-Campa et al. 2006). To our knowledge, this has not been demonstrated before. Immature animals exposed to U in drinking water had increased uterine weight and uterine luminal epithelial cell growth, selective reduction of ovarian primary follicles but more growing follicles, accelerated VO, and persistent presence of cornified vaginal cells. U-mediated responses were blocked by coadministration of the antiestrogen ICI 181,720, indicating that an activated ER was necessary. In addition, transplacental exposure to U caused fewer primordial follicles in developing pup ovaries. These observations support the conclusion that U acts like estrogen in the female mouse reproductive tract.

U caused estrogenic responses at or below the U.S. EPA safe drinking water level of 30 µg/L (0.126 µM) (U.S. EPA 2006). The U.S. EPA safe drinking water level equals the concentration of elemental U and is 47.4% of UN dissolved in water. Therefore, the highest UN concentration of 60 mg/L equals 28 mg/L of elemental U. At first, we used milligram per liter amounts of UN in the drinking water because we expected U to cause ovarian chemical toxicity as previously reported (Maynard and Hodge 1949). Unexpectedly, at milligram per liter concentrations, U targeted only large primary follicles, causing a reduction in their number but an increase in growing follicles. At the same time, there was a trend of increasing uterine weight with increasing U dose. These results led us to determine whether U could mimic estrogen's effects on the female reproductive system. Subsequently, we analyzed uterotrophic responses in ovariectomized mice using environmentally relevant U concentrations. We observed significant effects of U on the female reproductive system at or below the U.S. EPA safe levels.

The U levels used in these experiments are well within the range of U concentrations measured in numerous water sources on the Navajo Reservation, where concentrations > 1 mg/L have been reported (Brugge and Goble 2002; U.S. EPA 2004). The Navajo Reservation is a vast expanse of primarily rural and open range land. At least half of the households on the Navajo Reservation rely on water hauled from the nearest source for household use (U.S. Census 2006). Given the frequency of water supplies with unsafe U content, there is no doubt that many of the 175,000 residents living on the Navajo Reservation are exposed to hazardous levels of U in their water (Brugge et al. 2007; Pasternak 2006).

Adult mice exposed to U while immature had fewer primary follicle populations but more secondary follicles. 17β-Estradiol (E_2) inhibits mouse oocyte nest breakdown and follicle assembly (Chen et al. 2007). U, mimicking E_2 action, may have reduced follicle assembly leading to fewer primary follicles. Dam ovaries had fewer small primary follicles at a 1,000-fold lower U concentration than did the adult nonpregnant mice, which had no significant decrease in primary follicles. The pregnant dam ovaries may have been more sensitive to U because of an up-regulation of ERs that occurs during pregnancy (Spong et al. 2000). Estrogen prevents early follicle assembly (Chen et al. 2007) but stimulates secondary or growing follicles (Drummond 2006). U exposure may have reduced primary follicle populations and stimulated growing follicles via its estrogen-like activity.

Developing embryos are exquisitely sensitive to chemical influences. U concentrations of 0.001 or 0.120 µM in the dams' drinking water led to a significant reduction in the number of primordial follicles in pup ovaries. Gestational DES exposure is linked to fewer primordial follicles in pups, resulting in fewer ovulated ova (McLachlan et al. 1982). The long-term consequence of fewer primordial follicles would lead to accelerated ovarian failure, resulting in an earlier menopause onset (Chen et al. 2007). The change in pup ovary primordial follicles with uranium dose was an inverted U-shaped curve. Inverted U-shaped curves are seen in responses resulting from *in utero* exposure to E_2 (Welshons et al. 2003).

The rodent uterotrophic assay is used to identify putative EDCs. Exposure to chemicals

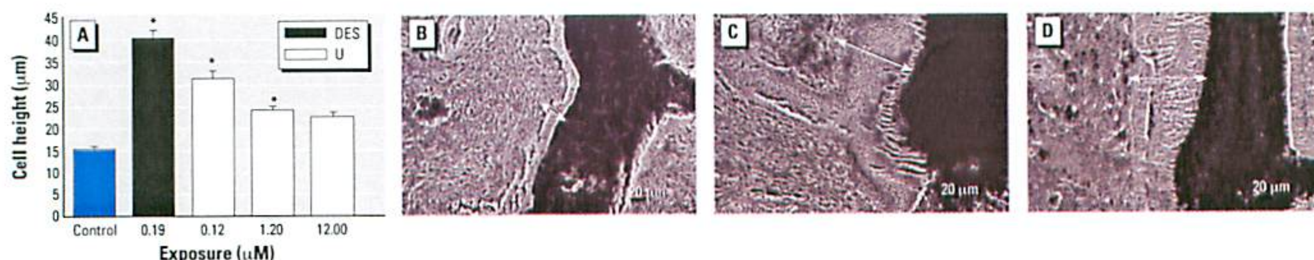

Figure 3. Uterine luminal epithelial cell growth in ovariectomized C57Bl/6J mice stimulated by UN or DES in drinking water for 30 days. (*A*) Cell height in uteri collected and prepared for scanning electron microscopy; values shown are mean ± SE (*n* = 5 uteri at 40 measurements from each tissue). Representative scanning electron microscopy images at the same magnification of uterine epithelial cell layers from tap water control (*B*), 0.19 µM DES (*C*), or 0.12 µM U (*D*). Arrows highlight epithelial cell height in DES-exposed (*C*) and U-exposed (*D*) ovariectomized mice.
*Significantly different compared with control ($p < 0.0001$).

with estrogenic activity are analyzed in immature rodents or ovariectomized mature rodents (Markey et al. 2001; Owens and Ashby 2002; Padilla-Banks et al. 2001). In our first experiment, the mice were immature at the outset but became sexually mature during the 30-day exposure to U. These mice exhibited a trend of increased uterine weight. If these mice had been examined for estrous stage at sacrifice, the uterine weights could have been grouped by stage, possibly enabling the trend to reach statistical significance. We used ovariectomized mice to avoid the confounding effect of estrous cycling to test whether UN caused uterotrophic responses.

The uterotrophic assay measures the consequences of three coordinated responses to estrogen or a chemical that acts like estrogen: epithelial cell growth, hyperemia, and fluid accumulation or imbibition (O'Brien et al. 2006). DES stimulation of uterine epithelial cell growth, in addition to employing classical ER-α, may also use tethered or nonclassical pathways to induce mitogenic uterine responses (O'Brien et al. 2006). This suggests that U does not need to directly activate the classical ER for uterine epithelial cell growth.

The U dose response was not monotonic in either the uterotrophic assay or in increased uterine epithelial cell height. Many EDCs elicit low-dose responses resulting in U-shaped or inverted U-shaped dose–response curves (Myers and Hessler 2007; Welshons et al. 2003). Nonmonotonic response occurs when a xenoestrogenic compound exerts direct effects by mimicking estradiol or indirect effects by interfering with ERs or estradiol production and metabolism. Further, xenoestrogenic responses may activate or inhibit different genes at various doses, which may result in different outcomes for target end points examined at the same time points (Coser et al. 2003).

Mice exposed to U for 30 days had a more pronounced uterotrophic response than mice exposed for 10 days. This raises questions about how U may be getting into cells/tissues and by which mechanism U interacts with the ER. U enters brain endothelial cells (Dobson et al. 2006), and via specialized transport it enters polarized epithelial LLC-PK_1 cells (Muller et al. 2006). Vidaud et al. (2007) examined the possibility of apotransferrin transporting U into the cell. U binds to transferrin, but conformational changes do not enable transferrin receptor recognition of the U-transferrin complex, ruling out this pathway for U to enter the cell. Other ways that U may enter the cell have not been investigated: divalent metal transporter-1 (DMT-1) or calcium channels. DMT-1 functions to transport iron and other metal ions across the plasma membrane, and is ubiquitous in plants, insects, microorganisms, and vertebrates (Mims and Prchal 2005). U displaces calcium in the bone matrix (Neuman et al. 1949); therefore, it is plausible that U may use calcium channels to enter the cell. The manner and rate by which U gets into the cell may be impeded by U speciation or tissue concentration, which could result in delayed responses, as we observed with uterine weight changes after 10-day exposure compared with 30-day exposure.

Similar to DES, U accelerated VO and stimulated persistent vaginal cornified cells, which represents a constant estrus state elicited by estrogen. U-stimulated uterine and vaginal responses were blocked by ICI 182,780, indicating that ER activation was necessary but not sufficient for U to act. We have yet to define the molecular mechanisms of action by which U evokes estrogenic responses. It is possible that U may elicit estrogen-like responses as Cd is reported to, by binding the ligand binding domain of the ER (Stoica et al. 2000). As mentioned above, U estrogenic stimulation may be the result of U binding some other factor whose responses are "tethered" to the ER pathway, resulting in cross-talk that induces estrogenic responses. In summary, the stimulatory effects of U on cells of the the ovary, uterus, and vagina suggest that U acts like estrogen in the female reproductive system and is an EDC.

There are few reports relating environmental U exposure to reproductive health outcomes in the Four Corners region. However, in one study, a statistically significant relationship was found between birth defects and the mother's proximity to U tailings (Shields et al. 1992). In another study, the incidence of reproductive or gonadal cancer in New Mexico Native American children and teenagers is 8-fold greater than that in age-matched non-Native American individuals (Duncan et al. 1986). Environmental estrogens such as DES or bisphenol A may contribute to occurrence of reproductive anomalies and cancer later in life (Maffini et al. 2006; Newbold et al. 2006). Given our results that U is an EDC, health problems may result from inappropriate concentration or timing of exposure to this estrogen mimic.

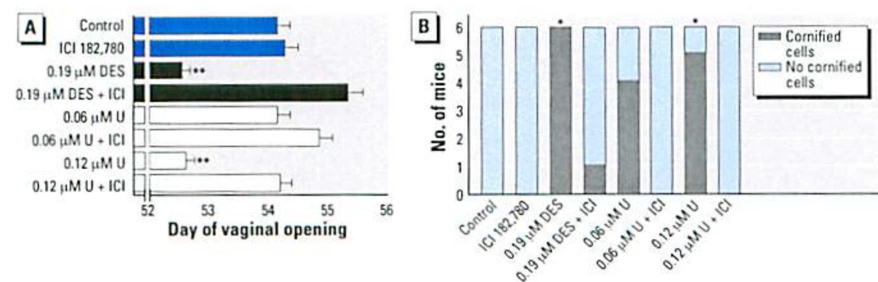

Figure 4. Effect of UN in drinking water on VO and presence of cornified vaginal cells. Ovariectomized C57Bl/6J mice (50 days of age) were exposed to control tap water, 0.19 µM DES, or 0.06 or 0.12 µM U for 10 days, or one of these doses plus vehicle or 500 µg/kg ICI 182,780 in vehicle. (*A*) Mice were examined daily for VO from 50 days of age to the day of vaginal opening; values shown are mean day of VO ± SE (n = 6–7). (*B*) Vaginal cell cornification determined from vaginal smears collected daily; the presence and absence of vaginal cornified cells were analyzed by chi-square test ($p < 0.05$).

*Statistically significant compared with control ($p < 0.05$ by Fisher's exact test). **Significantly different from control ($p < 0.001$).

References

Alonso-González C, González A, Mazarrasa O, Güezmes A, Sánchez-Mateos S, Martinez-Campa C, et al. 2007. Melatonin prevents the estrogenic effects of sub-chronic administration of cadmium on mice mammary glands and uterus. J Pineal Res 42:403–410.

Ashby J, Owens W, Odum J, Tinwell H. 2003. The intact immature rodent uterotrophic bioassay: possible effects on assay sensitivity of vomeronasal signals from male rodents and strain differences. Environ Health Perspect 111:1568–1570.

Ball H. 1993. Cancer factories: America's tragic quest for uranium self-sufficiency. Contrib Med Stud 37:1–188.

Brama M, Gnessi L, Basciani S, Cerulli N, Politi L, Spera G, et al. 2007. Cadmium induces mitogenic signaling in breast cancer cell by an ERα-dependent mechanism. Mol Cell Endocrinol 264:102–108.

Brugge D, de Lemos JL, Bui C. 2007. The Sequoyah Corporation fuels release and the Church Rock spill: unpublicized nuclear releases in American Indian communities. Am J Public Health 97:1595–1600.

Brugge D, deLemos JL, Oldmixon B. 2005. Exposure pathways and health effects associated with chemical and radiological toxicity of natural uranium: a review. Rev Environ Health 20:177–193.

Brugge D, Goble R. 2002. The history of uranium mining and the Navajo people. Am J Public Health 92:1410–1419.

Chen Y, Jefferson WN, Newbold RR, Padilla-Banks E, Pepling ME. 2007. Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary *in vitro* and *in vivo*. Endocrinology 148:3580–3590.

Choe SY, Kim SJ, Kim HG, Lee JH, Choi Y, Lee Y, et al. 2003. Evaluation of estrogenicity of major heavy metals. Sci Total Environ 312:15–21.

Coser KR, Chesnes J, Hur J, Ray S, Isselbacher KJ, Shioda T. 2003. Global analysis of ligand sensitivity of estrogen inducible and suppressible genes in MCF7/BUS breast cancer cells by DNA microarray. Proc Natl Acad Sci USA 100: 13994–13999.

Department of Energy. 2005. Remediation of the Moab Uranium Mill Tailings, Grand and San Juan Counties, Utah, Final Environmental Impact Statement. DOE/EIS-0355. Available: http://www.eh.doe.gov/nepa/eis/eis0355 [accessed 29 August 2007].

Dobson AW, Lack AK, Erikson KM, Aschner M. 2006. Depleted uranium is not toxic to rat brain endothelial (RBE4) cells. Biol Trace Elem Res 110:61–72.

Domingo JL. 2001. Reproductive and developmental toxicity of natural and depleted uranium: a review. Reprod Toxicol 15:603–609.

Drummond AE. 2006. The role of steroids in follicular growth. Reprod Biol Endocrinol 4:16; doi:10.1186/1477-7827-4-16 [Online 10 April 2006].

Duncan MH, Wiggins CL, Samet JM, Key CR. 1986. Childhood cancer epidemiology in New Mexico's American Indians,

Hispanic whites, and non-Hispanic whites, 1970–82. J Natl Cancer Inst 76:1013–1018.

Dyer CA. 2007. Heavy metals as endocrine disrupting chemicals. In: Endocrine-Disrupting Chemicals: From Basic Research to Clinical Practice (Gore AC, ed). Totowa, NJ:Humana Press, 111–133.

Gilman AP, Villeneuve DC, Secours VE, Yagminas AP, Tracy BL, Quinn JM, et al. 1998. Uranyl nitrate: 28-day and 91-day toxicity studies in the Sprague-Dawley rat. Toxicol Sci 41:117–128.

Gordon MN, Osterburg HH, May PC, Finch CE. 1986. Effective oral administration of 17β-estradiol to female C57Bl/6J mice through drinking water. Biol Reprod 35:1088–1095.

Guével RL, Petit FG, Goff PL, Métivier R, Valotaire Y, Pakdel F. 2000. Inhibition of rainbow trout (*Oncorhynchus mykiss*) estrogen receptor activity by cadmium. Biol Reprod 63:259–266.

Hedaya MA, Birkenfeld HP, Kathren RL. 1997. A sensitive method for the determination of uranium in biological samples utilizing kinetic phosphorescence analysis (KPA). J Pharm Biomed Anal 15:1157–1165.

Hindin R, Brugge D, Panikkar B. 2005. Teratogenicity of depleted uranium aerosols: a review from an epidemiological perspective. Environ Health 4:17; doi:10.1186/1476-069X-4-17 [Online 26 August 2005].

Institute of Laboratory Animal Research. 1996. Guide for the Care and Use of Laboratory Animals. Washington, DC:National Academy Press.

Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepko G, et al. 2003. Cadmium mimics the *in vivo* effects of estrogen in the uterus and mammary gland. Nat Med 9:1081–1084.

Kang YH, Anderson WA, DeSombre ER. 1975. Modulation of uterine morphology and growth by estradiol-17β and an estrogen antagonist. J Cell Biol 64:682–691.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162–11166.

Maffini MV, Rubin BS, Sonnenschein C, Soto AM. 2006. Endocrine disruptors and reproductive health : the case of bisphenol-A. Mol Cell Endocrinol 254–255:179–186.

Markey CM, Michaelson CL, Veson EC, Sonnenschein C, Soto AM. 2001. The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A. Environ Health Perspect 109:55–60.

Martin MB, Reiter R, Pham T, Avellanet YR, Camara J, Lahm M, et al. 2003. Estrogen-like activity of metals in MCF-7 breast cancer cells. Endocrinology 144:2425–2436.

Martinez-Campa C, Alonso-González C, Mediavilla MD, Cos S, González A, Ramos S. 2006. Melatonin inhibits both ERα activation and breast cancer cell proliferation induced by a metalloestrogen, cadmium. J Pineal Res 40:291–296.

Mayer LP, Devine PJ, Dyer CA, Hoyer PB. 2004. The follicle-deplete mouse ovary produces androgen. Biol Reprod 71:130–138.

Maynard EA, Hodge HC. 1949. Studies of the toxicity of various uranium compounds when fed to experimental animals. In: Pharmacology and Toxicology of Uranium Compounds (Voegtlin C, Hodge HC, eds.). New York:McGraw-Hill Book Company, Inc., 309–376.

McLachlan JA, Newbold RR, Shah HC, Hogan MD, Dixon RL. 1982. Reduced fertility in female mice exposed transplacentally to diethylstilbestrol (DES). Fertil Steril 38:364–371.

Mims MP, Prchal JT. 2005. Divalent metal transporter 1. Hematology 10:339–345.

Moure-Eraso R. 1999. Observational studies as human experimentation: the uranium mining experience in the Navajo Nation (1947–66). N Solutions: J Environ Occup Health Policy 9:163–178.

Muller D, Houpert P, Cambar J, Hengé-Napoli M-H. 2006. Role of the sodium-dependent phosphate co-transporters and of the phosphate complexes of uranyl in the cytotoxicity of uranium in LLC-PK_1 cells. Toxicol Appl Pharmacol 214:166–177.

Myers P, Hessler W. 2007. Does "the Dose Make the Poison"? Extensive Results Challenge a Core Assumption in Toxicology. Available: http://www.environmentalhealthnews.org/sciencebackground/2007/2007-0415nmdrc.html [accessed 29 August 2007].

Neuman WF, Neuman MW, Main ER, Mulryan BJ. 1949. The deposition of uranium in bone. VI. Ion competition studies. J Biol Chem 179:341–348.

Newbold RR, Padilla-Banks E, Jefferson WN. 2006. Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. Endocrinology 147:S11–S17.

Oak Ridge National Laboratory. 1998. Tailings Pile Seepage Model The Atlas Corporation Moab Mill, Moab, Utah. Grand Junction, CO:Oak Ridge National Laboratory, Environmental Technology Section. Available: http://www.ornl.gov/~webworks/cppr/y2001/rpt/112413.pdf [accessed 29 August 2007].

O'Brien JE, Peterson TJ, Tong MH, Lee EJ, Pfaff LE, Hewitt SC, et al. 2006. Estrogen-induced proliferation of uterine epithelial cells is independent of estrogen receptor α binding to classical estrogen response elements. J Biol Chem 281:26683–26692.

Office of Surface Mining. 1977. Surface Mining Control and Reclamation Act of 1977. Public Law 95-87, Available: http://www.osmre.gov/smcra.htm [accessed 29 August 2007].

Owens JW, Ashby J. 2002. Critical review and evaluation of the uterotrophic bioassay for the identification of possible estrogen agonists and antagonists: in support of the validation of the OECD uterotrophic protocols for the laboratory rodent. Crit Rev Toxicol 32:445–520.

Padilla-Banks E, Jefferson WN, Newbold RR. 2001. The immature mouse is a suitable model for detection of estrogenicity in the uterotrophic assay. Environ Health Perspect 109:821–826.

Panikkar B, Brugge D. 2007. The ethical issues in uranium mining research in the Navajo Nation. Account Res 14:121–153.

Pasternak J. 2006. Blighted homeland. A peril dwelt among the Navajos. Los Angeles Times (Los Angeles, CA), 19 November. Available: http://www.latimes.com/news/printedition/front/la-na-navajo19nov19,1,4865021,full.story [accessed 4 September 2007].

Paternain JL, Domingo JL, Ortega A, Llobet JM. 1989. The effects of uranium on reproduction, gestation, and postnatal survival in mice. Ecotoxicol Environ Saf 17:291–296.

Roscoe RJ, Deddens JA, Salvan A, Schnorr TM. 1995. Mortality among Navajo uranium miners. Am J Public Health 85:535–540.

Shields LM, Wiese WH, Skipper BJ, Charley B, Benally L. 1992. Navajo birth outcomes in the Shiprock uranium mining area. Health Phys 63:542–551.

Silva E, Lopez-Espinosa MJ, Molina-Molina JM, Fernandez M, Olea N, Kortenkamp A. 2006. Lack of activity of cadmium in *in vitro* estrogenicity assays. Toxicol Appl Pharmacol 216:20–28.

Spong CY, McCune SK, Sternberg EM, Gustafsson JA. 2000. Maternal estrogen receptor-β expression during mouse gestation. Am J Reprod Immunol 44:249–252.

Stoica A, Katzenellenbogen BS, Martin MB. 2000. Activation of estrogen receptor-β by the heavy metal cadmium. Mol Endocrinol 14:545–553.

Taylor DM, Taylor SK. 1997. Environmental uranium and human health. Rev Environ Health 12:147–157.

U.S. Census Bureau. 2006. We the People: American Indians and Alaska Natives in the United States. Available: http://www.census.gov/prod/2006pubs/censr-28.pdf [accessed 4 September 2007].

U.S. EPA (U.S. Environmental Protection Agency). 2004. Abandoned Uranium Mines on the Navajo Nation, Arizona. EPA Region 9. Available: http://yosemite.epa.gov/r9/sfund/overview.nsf/0/d502c488f1841dc488256aee007c11bc?OpenDocument [accessed 4 September 2007].

U.S. EPA (U.S. Environmental Protection Agency). 2006. Basic Information about Radionuclides in Drinking Water. Available: http://epa.gov/safewater/radionuclides/basicinformation.html [accessed 4 September 2007].

Vidaud C, Gourion-Arsiquaud S, Rollin-Genetet F, Tome-Celer C, Plantevin S, Pible O et al. 2007. Structural consequences of binding of UO_2^{2+} to apotransferrin: can this protein account for entry of uranium into human cells? Biochemistry 46:2215–2226.

Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS. 2003. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ Health Perspect 111:994–1006.

Table 7 supplement. Incidence rates and 95% confidence intervals of the 15 top cancers for American Indian/Alaska Native males by IHS region, CHSDA counties in selected areas in the United States, 1999-2004

	U.S. (CHSDA counties)				Northern Plains				Alaska			
Cancer site [a]	**Rank**	**Rate**[c]	**95%CI**	**Count**	**Rank**	**Rate**[c]	**95%CI**	**Count**	**Rank**	**Rate**[c]	**95%CI**	**Count**
All sites[b]		406.9	398.2 - 415.8	9,770		611.4	574.8 - 649.5	1,374		543.3	504.7 - 583.8	916
Prostate	1	104.0	99.4 - 108.6	2,264	1	164.6	145.0 - 185.8	325	3	82.1	67.2 - 99.2	130
Lung and bronchus	2	68.0	64.4 - 71.8	1,504	2	115.5	99.4 - 133.1	229	1	116.5	99.0 - 135.9	187
Colon and rectum	3	51.5	48.4 - 54.7	1,193	3	79.8	67.1 - 93.9	185	2	98.5	82.6 - 116.4	163
Kidney and renal pelvis	4	22.7	20.8 - 24.7	612	4	29.7	23.2 - 37.3	85	5	28.6	20.7 - 38.3	52
Urinary bladder	5	16.1	14.3 - 18.0	350	5	27.6	19.5 - 37.5	51	6	23.0	15.1 - 33.1	34
Non-Hodgkin lymphoma	6	14.9	13.3 - 16.6	387	8	20.2	13.8 - 28.1	45	11	13.2	8.6 - 19.3	30
Stomach	7	14.2	12.6 - 16.0	323	9	16.7	11.3 - 23.6	38	4	34.6	24.8 - 46.6	52
Oral cavity and pharynx	8	13.0	11.6 - 14.5	351	6	21.5	15.1 - 29.4	47	8	20.9	14.8 - 28.6	42
Liver and IBD	9	13.0	11.5 - 14.5	337	7	21.0	14.8 - 28.8	48	9	17.2	10.4 - 26.3	26
Leukemia	10	10.6	9.3 - 12.0	333	11	13.7	9.6 - 19.1	45	12	8.4	4.8 - 13.7	21
Pancreas	11	9.7	8.4 - 11.2	226	13	9.9	6.0 - 15.2	27	7	21.4	14.2 - 30.5	34
Esophagus	12	7.8	6.6 - 9.1	178	10	14.1	8.8 - 21.0	27	10	13.2	7.8 - 20.8	20
Myeloma	13	6.5	5.4 - 7.6	152	14	7.1	3.9 - 11.7	18	d	d	d	d
Melanoma of the skin	14	5.9	4.9 - 6.9	156	d	d	d	d	d	d	d	d
Larynx	15	5.3	4.3 - 6.4	129	12	10.3	5.7 - 16.6	22	d	d	d	d
Brain and ONS	16	5.0	4.2 - 5.9	170	15	5.6	3.1 - 9.3	20	d	d	d	d
Testis	17	4.0	3.5 - 4.7	188	17	5.1	3.3 - 8.0	25	13	6.1	3.7 - 10.0	19
Gallbladder	18	2.5	1.8 - 3.3	50	d	d	d	d	d	d	d	d

	Southern Plains				Pacific Coast				East			
Cancer site [a]	**Rank**	**Rate**[c]	**95%CI**	**Count**	**Rank**	**Rate**[c]	**95%CI**	**Count**	**Rank**	**Rate**[c]	**95%CI**	**Count**
All sites[b]		568.2	547.6 - 589.4	3,258		333.0	316.5 - 350.1	1,942		286.3	255.8 - 319.0	398
Prostate	1	156.9	145.9 - 168.5	838	1	83.1	74.8 - 91.9	446	1	82.9	66.7 - 101.5	107
Lung and bronchus	2	108.7	99.7 - 118.2	605	2	56.5	49.5 - 64.1	288	2	45.4	33.7 - 59.5	60
Colon and rectum	3	68.9	61.6 - 76.7	370	3	44.0	37.9 - 50.7	231	3	35.5	25.1 - 48.4	46
Kidney and renal pelvis	4	25.2	21.4 - 29.5	166	4	14.8	11.7 - 18.5	99	5	15.1	8.9 - 23.8	22
Urinary bladder	5	24.8	20.5 - 29.7	130	5	13.3	10.1 - 17.0	73	4	20.4	12.5 - 30.9	26
Non-Hodgkin lymphoma	6	23.5	19.5 - 28.1	136	8	12.6	9.7 - 16.1	82	d	d	d	d
Stomach	12	10.2	7.7 - 13.1	61	9	11.8	8.7 - 15.6	59	d	d	d	d
Oral cavity and pharynx	7	18.5	15.1 - 22.4	117	7	12.6	9.8 - 15.8	88	6	9.8	5.5 - 16.1	17
Liver and IBD	11	11.1	8.6 - 14.2	69	6	12.7	9.9 - 16.0	88	d	d	d	d
Leukemia	8	17.2	13.9 - 20.9	117	10	9.3	6.8 - 12.3	64	d	d	d	d
Pancreas	9	12.3	9.4 - 15.7	68	12	6.6	4.4 - 9.4	38	d	d	d	d
Esophagus	14	8.5	6.1 - 11.4	48	11	7.5	5.2 - 10.3	43	d	d	d	d
Myeloma	15	8.3	6.0 - 11.0	48	14	4.6	2.9 - 6.9	27	d	d	d	d
Melanoma of the skin	10	11.6	9.0 - 14.8	73	13	5.6	3.7 - 8.1	36	d	d	d	d
Larynx	13	9.1	6.8 - 12.0	55	17	3.2	1.9 - 5.1	22	d	d	d	d
Brain and ONS	16	8.1	6.1 - 10.6	61	16	3.7	2.4 - 5.5	33	d	d	d	d
Testis	17	3.5	2.4 - 5.0	34	15	3.8	2.8 - 5.2	45	d	d	d	d
Gallbladder	d	d	d	d	d	d	d	d	d	d	d	d

Table 7 supplement. Incidence rates and 95% confidence intervals of the 15 top cancers for American Indian/Alaska Native males by IHS region, CHSDA counties in selected areas in the United States, 1999-2004

Cancer site [a]	Southwest			
	Rank	Rate[c]	95%CI	Count
All sites[b]		261.5	249.0 - 274.5	1,882
Prostate	1	67.0	60.4 - 74.0	418
Lung and bronchus	4	22.1	18.4 - 26.4	135
Colon and rectum	2	26.7	22.8 - 30.9	198
Kidney and renal pelvis	3	25.1	21.4 - 29.1	188
Urinary bladder	11	5.5	3.7 - 7.7	36
Non-Hodgkin lymphoma	7	10.4	8.2 - 13.1	84
Stomach	5	16.0	12.9 - 19.5	106
Oral cavity and pharynx	12	4.8	3.3 - 6.7	40
Liver and IBD	6	12.6	10.1 - 15.6	95
Leukemia	10	6.7	5.0 - 8.7	76
Pancreas	8	7.6	5.6 - 10.0	52
Esophagus	13	4.4	2.9 - 6.4	30
Myeloma	9	7.4	5.4 - 9.9	48
Melanoma of the skin	17	3.6	2.3 - 5.4	26
Larynx	d	d	d	d
Brain and ONS	16	3.8	2.6 - 5.5	39
Testis	14	4.2	3.2 - 5.6	61
Gallbladder	15	4.1	2.6 - 6.1	26

SEER = Surveillance, Epidemiology, and End Results; NPCR = National Program of Cancer Registries; NAACCR = North American Association of Central Cancer Registries; IHS = Indian Health Service; CHSDA = IHS Contract Health Services Delivery Area; CI = confidence interval; IBD = intrahepatic bile duct; ONS = other nervous system.

Source: SEER and NPCR areas reported by NAACCR as meeting high-quality data standards from 1999 through 2004.

[a] Cancers are sorted in descending order according to sex-specific rates for AI/AN. More than 15 cancers may appear to include the top 15 cancers in every IHS region.

[b] All sites excludes myelodysplastic syndromes and borderline tumors.

[c] Rates are per 100,000 persons and were age-adjusted to the 2000 U.S. standard population (19 age groups - Census p25-1130).

[d] Statistic could not be calculated when fewer than 16 cases were reported.

Years of data and registries used (30 states)

1999-2004: Alabama, Alaska, California, Colorado, Connecticut, Florida, Idaho, Indiana, Iowa, Louisiana, Maine, Massachusetts, Montana, Nebraska, Nevada, New Mexico, New York, North Dakota, Oklahoma, Oregon, Pennsylvania, Rhode Island, South Carolina, Texas, Utah, Washington, Wyoming;
1999-2003: Arizona, Wisconsin; 2000-2004: Michigan.

Percent regional coverage of AI/AN CHSDA data to all AI/AN in region: Alaska - 100%, East - 12.2%, N. Plains - 38.1%, Pacific Coast - 55.1%, S. Plains - 65.0%, Southwest - 86.1%

Table 8 supplement. Incidence rates and 95% confidence intervals of the 15 top cancers for American Indian/Alaska Native females by IHS region, CHSDA counties in selected areas in the United States, 1999-2004

	U.S. (CHSDA counties)				Northern Plains				Alaska			
Cancer site [a]	Rank	Rate[c]	95%CI	Count	Rank	Rate[c]	95%CI	Count	Rank	Rate[c]	95%CI	Count
All sites[b]		334.8	328.3 - 341.5	10,563		468.1	442.4 - 494.7	1,400		511.0	479.8 - 543.6	1,084
Breast	1	84.7	81.5 - 88.0	2,807	1	112.2	100.5 - 124.8	368	1	139.5	124.3 - 156.0	325
Lung and bronchus	2	48.0	45.4 - 50.7	1,377	2	97.4	85.5 - 110.4	263	3	78.9	66.4 - 92.9	148
Colon and rectum	3	41.6	39.2 - 44.1	1,224	3	60.4	51.1 - 70.9	163	2	106.2	91.8 - 122.1	207
Corpus and uterus, NOS	4	18.2	16.7 - 19.8	605	4	21.3	16.1 - 27.4	64	5	14.0	9.5 - 19.9	32
Kidney and renal pelvis	5	13.9	12.6 - 15.3	441	5	18.8	14.0 - 24.6	57	8	12.0	7.7 - 17.6	26
Non-Hodgkin lymphoma	6	12.8	11.5 - 14.2	378	6	16.2	11.6 - 21.9	45	10	9.9	6.1 - 15.1	22
Ovary	7	11.7	10.5 - 13.0	383	7	12.6	8.8 - 17.4	40	12	7.6	4.3 - 12.3	17
Pancreas	8	9.4	8.3 - 10.7	263	11	9.9	6.3 - 14.6	26	9	11.9	7.5 - 17.8	24
Cervix uteri	9	9.4	8.4 - 10.4	353	9	11.3	8.1 - 15.4	44	11	9.2	5.7 - 14.0	23
Thyroid	10	8.4	7.5 - 9.4	338	10	10.2	7.2 - 14.1	42	6	12.6	8.8 - 17.7	36
Leukemia	11	7.8	6.8 - 8.9	275	8	11.5	7.5 - 16.7	31	14	5.7	3.1 - 9.5	16
Stomach	12	7.6	6.5 - 8.7	216	14	7.4	4.5 - 11.3	22	4	17.7	12.2 - 24.7	36
Liver and IBD	13	5.9	5.0 - 6.9	164	13	7.7	4.5 - 12.1	19	d	d	d	d
Myeloma	14	5.8	4.9 - 6.7	172	15	5.6	3.2 - 9.0	17	13	7.5	4.2 - 12.3	16
Oral cavity and pharynx	15	4.9	4.2 - 5.8	160	12	9.6	6.3 - 13.7	31	7	12.3	7.8 - 18.3	25
Urinary bladder	16	4.4	3.6 - 5.2	124	d	d	d	d	d	d	d	d
Melanoma of the skin	17	3.9	3.2 - 4.7	133	d	d	d	d	d	d	d	d
Gallbladder	18	3.8	3.1 - 4.6	104	d	d	d	d	d	d	d	d

	Southern Plains				Pacific Coast				East			
Cancer site [a]	Rank	Rate[c]	95%CI	Count	Rank	Rate[c]	95%CI	Count	Rank	Rate[c]	95%CI	Count
All sites[b]		439.2	424.3 - 454.5	3,343		292.7	279.6 - 306.1	2,141		258.3	234.4 - 283.9	457
Breast	1	115.2	107.7 - 123.1	894	1	74.5	68.2 - 81.1	579	1	69.1	57.4 - 82.4	131
Lung and bronchus	2	68.8	62.9 - 75.1	503	2	47.0	41.6 - 52.8	305	2	45.2	35.1 - 57.2	71
Colon and rectum	3	54.4	49.1 - 60.0	399	3	34.8	30.2 - 39.8	232	3	34.6	26.1 - 44.9	58
Corpus and uterus, NOS	4	22.5	19.2 - 26.1	172	4	16.8	13.9 - 20.0	133	4	13.1	8.2 - 19.8	23
Kidney and renal pelvis	6	17.9	15.0 - 21.2	134	7	10.4	8.1 - 13.1	78	5	11.5	7.1 - 17.5	22
Non-Hodgkin lymphoma	5	18.4	15.3 - 21.8	133	5	12.4	9.7 - 15.5	83	d	d	d	d
Ovary	7	14.4	11.8 - 17.3	111	8	9.9	7.6 - 12.5	76	d	d	d	d
Pancreas	9	9.9	7.7 - 12.4	71	6	10.8	8.3 - 13.8	67	d	d	d	d
Cervix uteri	8	14.1	11.7 - 16.9	118	10	6.9	5.2 - 8.9	65	6	8.2	4.8 - 13.1	18
Thyroid	11	9.3	7.3 - 11.6	81	11	6.3	4.8 - 8.2	61	7	7.9	4.5 - 12.8	17
Leukemia	10	9.7	7.6 - 12.1	82	9	7.5	5.6 - 9.8	60	d	d	d	d
Stomach	13	7.4	5.5 - 9.7	53	13	4.4	2.8 - 6.4	27	d	d	d	d
Liver and IBD	17	5.3	3.7 - 7.3	37	12	5.9	4.1 - 8.2	38	d	d	d	d
Myeloma	15	6.8	5.1 - 9.0	50	15	4.1	2.6 - 6.0	27	d	d	d	d
Oral cavity and pharynx	16	5.9	4.3 - 7.9	45	17	3.9	2.6 - 5.7	32	d	d	d	d
Urinary bladder	14	7.3	5.4 - 9.5	52	14	4.3	2.8 - 6.2	28	d	d	d	d
Melanoma of the skin	12	7.7	5.9 - 9.9	61	16	4.0	2.7 - 5.7	33	d	d	d	d
Gallbladder	20	2.6	1.6 - 4.1	19	d	d	d	d	8	0.0	.0 - 1.8	0

Table 8 supplement. Incidence rates and 95% confidence intervals of the 15 top cancers for American Indian/Alaska Native females by IHS region, CHSDA counties in selected areas in the United States, 1999-2004

Cancer site [a]	Southwest			
	Rank	Rate [c]	95%CI	Count
All sites [b]		219.3	209.7 - 229.1	2,138
Breast	1	50.4	46.0 - 55.0	510
Lung and bronchus	6	10.3	8.2 - 12.7	87
Colon and rectum	2	17.6	14.9 - 20.6	165
Corpus and uterus, NOS	3	16.8	14.4 - 19.5	181
Kidney and renal pelvis	5	12.6	10.4 - 15.1	124
Non-Hodgkin lymphoma	7	9.0	7.1 - 11.2	82
Ovary	4	13.1	10.9 - 15.6	130
Pancreas	11	7.9	6.0 - 10.1	65
Cervix uteri	10	7.9	6.3 - 9.9	85
Thyroid	9	8.4	6.8 - 10.3	101
Leukemia	14	6.1	4.7 - 7.8	74
Stomach	8	8.8	6.8 - 11.1	72
Liver and IBD	15	6.0	4.4 - 7.9	50
Myeloma	13	6.1	4.6 - 8.0	55
Oral cavity and pharynx	18	2.1	1.3 - 3.2	21
Urinary bladder	d	d	d	d
Melanoma of the skin	20	1.8	1.1 - 2.8	21
Gallbladder	12	6.6	4.9 - 8.7	52

SEER = Surveillance, Epidemiology, and End Results; NPCR = National Program of Cancer Registries; NAACCR = North American Association of Central Cancer Registries;
IHS = Indian Health Service; CHSDA = IHS Contract Health Services Delivery Area; CI = confidence interval; IBD = intrahepatic bile duct; NOS= not otherwise specified.
Source: SEER and NPCR areas reported by NAACCR as meeting high-quality data standards from 1999 through 2004.
a Cancers are sorted in descending order according to sex-specific rates for AI/AN. More than 15 cancers may appear to include the top 15 cancers in every geographic region.
[b] All sites excludes myelodysplastic syndromes and borderline tumors; ovary excludes borderline tumors.
[c] Rates are per 100,000 persons and were age-adjusted to the 2000 U.S. standard population (19 age groups - Census p25-1130).
[d] Statistic could not be calculated when fewer than 16 cases were reported.

Years of data and registries used (30 states)

1999-2004: Alabama, Alaska, California, Colorado, Connecticut, Florida, Idaho, Indiana, Iowa, Louisiana, Maine, Massachusetts, Montana, Nebraska, Nevada, New Mexico, New York, North Dakota, Oklahoma, Oregon, Pennsylvania, Rhode Island, South Carolina, Texas, Utah, Washington, Wyoming; 1999-2003: Arizona, Wisconsin; 2000-2004: Michigan.
Percent regional coverage of AI/AN CHSDA data to all AI/AN in region: Alaska - 100%, East - 12.2%, N. Plains - 38.1%, Pacific Coast - 55.1%, S. Plains - 65.0%, Southwest - 86.1%.