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EFFECTS OF HEAVY METALS ON IMMUNOCOMPETENCE OF WHITE-FOOTED MICE (*PEROMYSCUS LEUCOPUS*)

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ABSTRACT: Continued human population growth and industrialization result in increased contamination of wildlife habitats. Effects of such habitat deterioration on the well-being of natural populations are unclear. Exposure to contaminants may impair immunocompetence, thereby increasing disease susceptibility. The mammalian immune system is important in maintaining health and in its sensitivity to toxins. In our study conducted from May 1999 through May 2001, we examined assays of immunocompetence in the white-footed mouse (*Peromyscus leucopus*) that inhabited reference sites and sites significantly contaminated with mixtures of heavy metals. We estimated potential exposure and uptake of heavy metals by measuring the level of each contaminant in representative soil and tissue samples. Intraindividual variation across mice, but not sex, explained a large portion of the overall variance in immune response, and spleen weight was significantly affected by mouse age. We found no evidence that residence on contaminated sites had any effect on immunopathology and humoral immunity as measured in our study. We suggest that field and laboratory studies in ecotoxicology provide estimates of exposure to contaminants (i.e., tissue analyses) to establish a database suitable to clarify the dose-response relationship between contaminants and target systems.

Key words: Ecotoxicology, flow cytometry, heavy metal contamination, immunopathology.

INTRODUCTION

As continued human population growth and industrialization encroach upon what were once pristine habitats, natural populations of organisms are increasingly exposed to environmental pollutants. Laboratory studies have demonstrated that exposure to pollutants, such as heavy metals and polychlorinated biphenyls (PCBs), adversely impact a variety of mammalian target organ systems, including the immune system (Exon et al., 1985; Hilliam and Ozkan, 1986; Burchiel et al., 1987; Kowolenko et al., 1988; Koller, 1990; Goyer, 1991; Rocke and Samuel, 1991; McMurry et al., 1995; Johnson, 1998; Fair and Myers, 2002; Segre et al., 2002; Kim and Sharma, 2003). Immunocompetence is vital in maintaining the overall health of an organism and is extremely sensitive to toxins, such as heavy metals (Dean and Murray, 1991; McMurry et al., 1995; Institoris et al., 2001). The immune system is therefore a useful target for environmental risk assessment studies with wild animals.

There are numerous immunologic assays to evaluate immunocompetence in laboratory and wild animals (Dean and Murray, 1991; Lochmiller et al., 1994; Propst et al., 1999; Grasman and Fox, 2001; Institoris et al., 2001; Smits and Bortolotti, 2001). Measurements of spleen weight and lymphocyte number allow evaluation of the immunopathologic condition of some animals in response to chemical exposure (Dean and Murray, 1991; McMurry et al., 1994; Institoris et al., 2001; Grasman, 2002). Humoral immunity of laboratory animals is commonly assessed using spleen plaque-forming assays, which permit quantification of antibody production in response to challenge with sheep red blood cells (Luster et al., 1978; Institoris et al., 2001). Such assays may provide information regarding impacts, mechanisms of action, and dose-response relationships between contaminants and the immune system (Dean and Murray, 1991). Laboratory studies have been crucial in establishing assays that detect pollution-induced changes in immune responses and in identifying relevant con-

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taminants and potential harmful threshold levels of exposure. However, laboratory studies rarely reproduce the exposure levels and chemical interactions of mixtures of contaminants that are typically found in natural environments. In addition, they cannot reproduce ecologic conditions that influence exposure and uptake of these pollutants in the wild, such as biomagnification and bioaccumulation (McBee and Bickham, 1990). Laboratory studies, therefore, should be complimented by risk assessment studies in natural populations exposed to typical environmental contaminant mixtures and levels (Linzey, 1987; Propst et al., 1999). Ideally, such ecotoxicologic field studies should not only assay the immune response of wild animals, but also provide estimates of contaminant levels of soil and animal tissue. If both laboratory and field researchers provided tissue contaminant levels for animals monitored in toxicology studies, such studies could be more readily compared, thus enhancing our understanding of harmful threshold levels for mixtures or single chemicals. Lacking these congruent data sets, laboratory studies provide little guidance with respect to critical thresholds of contaminants relevant to wild animals. Every comprehensive risk assessment of environmental contaminants should have a field and experimental laboratory component to establish dose-response relationships between those contaminants and the endpoints of interest (e.g., immune system). However, reporting of relevant tissue contaminant levels in all toxicology studies might be the most efficient first step to make individual field and laboratory studies complimentary.

In our study, conducted from May 1999 through May 2001, we examined several assays of immunocompetence in white-footed mice (*Peromyscus leucopus*), that inhabited reference sites and sites significantly contaminated with mixtures of heavy metals (e.g., zinc, lead, cadmium, mercury). We estimated potential exposure and uptake of heavy metals by measuring the level of each contaminant in representative soil and tissue samples. White-footed mice are suitable biomonitors to assess the effects of pollutants on the immune response of wild mammals for a variety of reasons (Jenkins, 1981): they are 1) ubiquitous and abundant; 2) found naturally in pristine and polluted environments; and 3) key intermediates in the transfer of contaminants from soils to higher trophic levels (Laurinolli and Bendell-Young, 1996). In addition, they are large enough to provide sufficient tissue for multiple immunologic assays. We studied free-ranging white-footed mice potentially exposed to heavy metals. Specifically, we evaluated immunopathology based on spleen weight and lymphocyte enumeration using flow cytometry. We assessed humoral immunity using the spleen plaque-forming assay. In our statistical analysis we aimed to distinguish effects on the immune system that were due to interindividual variability (i.e., genetic background, sex, age, reproductive condition) and contaminant exposure.

MATERIALS AND METHODS

Study areas

The New Jersey Zinc/Mobil Chemical (NJZMC; 41°19′N, 89°19′E) locality is approximately 324 ha of land adjacent to the village of DePue in Bureau County, Illinois. Throughout much of the 20th century, various plants at the locality produced zinc and sulfuric acid. Previous contaminant assessment documented elevated levels of heavy metals (e.g., cadmium, copper, lead, mercury, zinc) in soil and sediment samples collected from NJZMC, as well as nearby village and private properties (Illinois Environmental Protection Agency, 1992). To assess risks associated with potential heavy metals exposure, we collected white-footed mice from four sites on village and private property (i.e., EWT, WWT, EAST, BB) in the immediate vicinity of NJZMC (Biser, 2001). We compared immunologic data from these animals with data from mice trapped from three sites at Goose Lake Prairie (GLP; i.e., GL1, GL2, GL3). GLP (41°22′N, 88°19′E) is located 82 km east of DePue and 0.6 km southwest of the confluence of the Kankakee and Des Plaines Rivers in Grundy County, Illinois. Because the habitat and general geographic location were compa-

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TABLE 1. Levels (mg/kg dry weight) of heavy metals present in soil and liver tissue samples from mice collected from the contaminated site (DePue) and reference sties (GL1, CS1), including results of Wilcoxon tests of significantly different mean concentrations for contaminants. Provided are mean±SE (mg/kg) and range of contaminant amounts.

	Soil		Liver tissue	
	DePue (n=10)	GL1 (n=10)	DePue (n=5)	CS1 (n=5)
Cadmium	38.6±11.34[a]	0.32±0.24	0.93±0.38[a]	0.04±0.01
	4.7–106	0.24–0.50	0.3–1.7	0.02–0.07
Copper	42.9±9.34[a]	14.50±0.48	6.22±0.35	5.58±0.34
	13–98	13–18	5.4–7.1	4.6–6.4
Lead	194.77±54.03[b]	23.10±0.71	0.24±0.08[a]	<0.05±0
	41–466	21–28	<0.05–0.4	<0.05
Mercury	0.046±0.007[a]	0.03±0.001	0.0022±0.0002[a]	0.0072±0.0007
	0.032–0.089	0.01–0.04	0.0014–0.0028	0.0049–0.0092
Zinc	2497.31±828.91[b]	59.00±2.04	27±1.7	23.8±0.92
	527–8020	47–67	25–33	22–27

[a] $P<0.05$ for comparison between contaminated and reference sites.
[b] $P<0.001$.

rable with that of NJZMC and Goose Lake Prairie has no known history of pollution, it was a suitable area for reference sites. In our analysis, we referred to any of the seven study sites as "site" and investigated effects of contamination on mice collected at contaminated (NJZMC) and reference (GLP) sites by examining the variable "type of site."

Field methods and heavy metals analysis of soil and tissue samples

We trapped white-footed mice at each of the reference and contaminated sites between May and October 2000 using Sherman live traps (H. B. Sherman Traps, Tallahassee, Florida, USA) baited with a mixture of peanut butter and oats. Traps were placed at 5-m intervals along parallel transects. We transported animals from the field to an outdoor quarantine facility at Illinois State University and strictly followed guidelines for handling rodents potentially infected with hantavirus (Mills et al., 1995). All animals were tested for hantavirus antibodies (Yee et al., 2003; Camaioni et al., 2001) and we only used clearly seronegative mice for our immunologic analyses. During the quarantine period we weighed all animals, determined sex, and estimated their ages based on secondary sexual characteristics. In addition, we aged mice more accurately postmortem by examining tooth morphology (Hoffmeister, 1951), which allowed us to place animals into one of five age classes.

In order to assess exposure potential to heavy metals, we collected five to 10 soil samples along parallel transects based on a stratified sampling approach. We chose the following subset of representative sites for this contaminant analysis: Goose Lake site 1 (GL1) for the reference sites, and DePue sites EWT and WWT for the contaminated sites (Table 1). We obtained soil using an 8×15-cm hand-held stainless steel core sampler in accordance with standard protocols (Illinois Department of Natural Resources, 1999). Homogenized soil samples were dried and subsequently analyzed for heavy metals by staff of the Research and Laboratory Services Program (RLSP) at the Illinois Department of Natural Resources Waste Management Resource Center (IDNR-WMRC). We chose those heavy metals for soil and tissue analysis that had previously been documented at the DePue Superfund Site (US Environmental Protection Agency [EPA] FRL-6338-5, 1999). Metals tested at the reference sites were, at a minimum, those that had been documented for its matched contaminated sites. Analyses for cadmium, copper, lead, and zinc were obtained by inductively coupled plasma mass spectroscopy (ICP-MS); mercury concentrations were determined using cold vapor atomic fluorescence (CVAF) detection. Samples were analyzed for heavy metals according to standard protocols used by the IDNR-WMRC (EPA, 1986, 1992). We report values for contaminant concentrations as mg/kg dry weight.

In order to assess contaminant uptake, and therefore an estimate of actual exposure, we analyzed tissue samples from mice collected at a subset of study sites for heavy metals as described above. At the time of sacrifice, the liver

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of each mouse was removed and stored at −80 C. Prior to heavy metal analysis, we prepared each specimen according to standard protocols (EPA, 1986, 1992). We report values as mg/kg wet weight. Appropriate quality controls were performed for all soil and tissue samples.

Because we had no tissue samples analyzed from mice captured at Goose Lake Prairie reference sites, we compared contaminant levels from five mouse livers derived from the DePue EWT and WWT contaminated sites with samples from five mice collected at a more southern reference site, called CS1. Reference site CS1 (37°37′N, 89°06′W) is located within Crab Orchard National Wildlife Refuge (CONWR), which is 504 km south of DePue in Williamson County, Illinois, and is part of our larger-scale effort to document biologic impacts at several Superfund Sites in Illinois. The CS1 reference site is located in the part of the refuge that historically has been free of industrial development and has been managed solely as a wildlife and recreation area since the creation of the refuge.

Immunologic assays

Animals: Mice were a subsample of a larger number of mice captured at our study sites; we used 62 mice (38 male and 24 female) from a total of three reference (GL1, GL2, GL3) and four contaminated (EWT, WWT, EAST, BB) sites. Field identification of age was not always reliable, and definitive analysis of age was based on tooth wear evaluated postmortem (Hoffmeister, 1951, 1989).

Immunization and spleen weight: Seven days prior to sacrificing each mouse, we administered an intraperitoneal injection of 20% sheep red blood cells (SRBC; Colorado Serum Company, Denver, Colorado, USA) in 0.85% physiologic saline to each mouse at a dose of 10 μl/g body weight. Negative control for each group of wild mice was one laboratory mouse (C57BL/6, National Cancer Institute, Bethesda, Maryland, USA) that had not been injected with SRBC. The spleen of each animal was collected at sacrifice and all fat and extraneous tissue was removed. Using an analytical scale (XS-310D, Denver Instrument Company, Arvada, Colorado, USA), we recorded weights to the nearest thousandth of a gram and expressed it as a percentage of total body weight (SPWT).

Spleen plaque-forming assay. We prepared a single cell suspension from each spleen by grinding the organ over a piece of wire mesh with a syringe stopper, subsequently washed the cells in 15 ml balanced salt solution (BSS), and centrifuged them for 5 min at 500 × G. After decanting the supernatant, red blood cells were lysed by resuspending the pellet in 3 ml of ammonium Tris chloride buffer (0.144 M NH_4Cl, 0.017 M Tris, pH 7.2) and subsequent incubation at 37°C. Total time of spleen cell exposure to ammonium Tris chloride buffer was 3 min. We again washed cells in 15 ml BSS, centrifuged as above, and resuspended them in 5 ml BSS. Lymphocytes were counted via hemocytometer using trypan blue dye (Sigma, St. Louis, Missouri, USA) exclusion. From each spleen cell suspension, we transferred three replicates of two separate cell concentrations (2.5×10^6 and 5×10^5) to microcentrifuge tubes and resuspended them in BSS-5% fetal calf serum (Fisher, Pittsburgh, Pennsylvania, USA) to a final volume of 100 μl. We then placed the spleen cells on ice. A 5-ml suspension of SRBC was washed three times with BSS and resuspended to 10% with BSS-5% fetal calf serum. We prepared a 0.5% solution of low melting point agarose (Sigma) in BSS and diluted guinea pig complement (Accurate Chemicals, Westbury, New York, USA) 1:10 in BSS. Glass tubes (12×75; Fisher) were incubated in a 37°C water bath for 15 min and the following were sequentially added to each tube: 0.5 ml agarose, 100 μl spleen cells, 50 μl SRBC, and 50 μl guinea pig complement. After gently vortexing, we poured the contents of each tube onto a slide that had been previously scored with a wax pencil to prevent liquid from spilling. All slides were incubated for 4 hr in a 37°C humidity chamber. We recorded the number of spleen plaque-forming cells (PFCs) per slide for each concentration.

The spleen plaque-forming assay is usually performed in triplicate at two different concentrations of spleen cells: 5×10^5 and 2.5×10^6 cells. However, lymphocyte numbers from animals used in this study were not always sufficient to replicate both concentrations three times. Therefore, we only scored plaques for those assays performed in triplicate at a concentration of 5×10^5 cells. A one-way analysis of variance (ANOVA) on log-transformed plaque counts showed that the variance across individuals, as compared to within individuals, accounted for the majority of variation (72.5%) in PFCs. Consequently, we averaged the three plaque count replicates and used the mean number of plaque-forming cells per mouse in all subsequent analyses.

Flow cytometry. After determining cell counts for each spleen suspension as described above, we transferred 5×10^5 additional cells per mouse to microcentrifuge tubes in single aliquots. We washed spleen cells and resuspended them in Hanks bovine serum albumin (Fisher). Five microliters of normal rat serum was added to all tubes to block nonspecific

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binding. To detect $CD3^+$, $CD4^+$, $CD8^+$ T lymphocytes, and B lymphocytes, we respectively added fluorescent stains (PharMingen, San Diego, California, USA) to each tube in the following optimal concentrations: 1) 2.5 μl PerCP-conjugated hamster anti-mouse CD3 monoclonal antibody; 2) 0.5 μl FITC-conjugated rat anti-mouse CD4 (L3T4) monoclonal antibody; 3) 0.5 μl R-PE-conjugated rat anti-mouse CD8a (Ly-2) monoclonal antibody; and 4) 1.0 μl FITC-conjugated rat anti-mouse B220 clone 6B2 monoclonal antibody. Spleen cells from a normal laboratory mouse (BALB/c) served as a control for the staining. We incubated cells on ice in the dark for 15 min, then washed and resuspended them in 300 μl Hanks bovine serum albumin. All samples were analyzed using a FACScalibur flow cytometer with a single argon (488 nm) laser (Becton, Dickinson, and Co., Franklin Lakes, New Jersey, USA). The cytometer was calibrated using cells from the laboratory mouse treated with 1) no stains added; 2) PerCP CD3; 3) FITC CD4; 4) PE CD8; 5) FITC B220; or 4) isotype controls (2.5 μl PerCP-conjugated hamster IgG, group 1, κ monoclonal immunoglobulin, 0.5 μl FITC-conjugated rate IgG_{2a} monoclonal immunoglobulin, 0.5 μl R-PE-conjugated rat IgG2a monoclonal immunoglobulin, and 1.0 μl FITC-conjugated rat IgG_{2a} monoclonal immunoglobulin; PharMingen). Using Cell Quest software (Becton, Dickinson, and Co.), we gated cells based on lymphocytes, and we recorded percent B cells for each mouse (BCELLS). We could not label T cells of our wild mice with monoclonal antibodies despite the fact that antibodies bound to T cells derived from the control laboratory mice.

Statistical analyses

We performed all analyses using Statistical Analytical Systems software (1996, SAS Institute, Inc., Cary, North Carolina, USA). Fisher's exact tests were used to determine if age or sex distribution differed significantly between animals collected from contaminated and reference sites (Sokal and Rohlf, 1995). Because the data on soil and tissue contaminant levels did not meet the assumptions for parametric analyses, we used Wilcoxon nonparametric tests to examine differences between contaminated and reference sites with respect to soil and tissue concentrations for cadmium, copper, lead, mercury, and zinc. We combined soil data obtained from the two contaminated sites (i.e., EWT, WWT) for comparison with the soil samples analyzed for reference site GL1.

In order to assess whether it was appropriate to use the mean number of PFCs per mouse in subsequent analyses, we performed a separate ANOVA to compare the variance of PFCs for the three replicates of cell counts within individuals to the variance across individuals. In addition, we calculated the Pearson product-moment correlation coefficients to assess the nature of the relationships between the three immunologic variables (BCELLS, PFC, SPWT).

In order to determine if immunologic data could be pooled across age classes, we performed ANOVAs using age, sex, type of site (reference or contaminated), and "site nested within type of site" as factors. Sex, age, and type of site (contaminated or reference) were treated as fixed effects, and specific site location (site) was nested within type of site and treated as a random effect. We subsequently analyzed the results using ANOVA and multivariate analysis of covariance (MANCOVA). The proportion of variance contributed by each factor in the model was computed using the VARCOMP procedure in SAS. We transformed percent B cells, PFC, and spleen weight data to meet the assumptions of parametric statistics (square root, log, and reciprocal transformed, respectively).

RESULTS

Soil and tissue analyses

Soil levels of heavy metals were significantly elevated at the combined contaminated DePue sites (EWT, WWT) for all five metals tested (Table 1). Tissue samples from the contaminated DePue sites contained significantly more cadmium and lead, but not mercury, when compared with samples from the Crab Orchard reference site (Table 1).

Immunologic assays

Mice ranged from age class 1 to 5, and age did not differ between contaminated and reference sites (Fisher's exact test, $P>0.05$). Spleen weights ranged from 0.095 to 0.966 g for animals from reference areas, and from 0.100 to 0.458 g for animals from contaminated areas. We detected a significant negative correlation between spleen weight and mean number of PFCs (Pearson correlation coefficient$=-0.29$, $P=0.03$). Consequently, we tested the effect of contamination on the

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TABLE 2. Results of the three-way analysis of variance (ANOVA) for spleen weight. Age had a significant interaction with type of site (contaminated or reference) for spleen weight.

Source	DF[a]	Type III MS[b]	Denominator DF[a]	Denominator MS[b]	*F*-value	Pr[c]>*F*	Percent of total variance
Age	4	8.23	4.76	3.65	2.51	0.20	3.49
Sex	1	0.74	3.5	3.20	0.25	0.66	0.85
Type of site	1	1.24	6.21	4.20	0.30	0.60	3.77
Age×sex	3	9.21	3.23	6.43	1.43	0.38	0.24
Age×type of site	3	21.36	5.55	3.92	5.45	0.04	6.52
Sex×type of site	1	0.25	1.83	3.48	0.07	0.81	5.35
Site (type)	5	3.49	0.01	0.53	6.56	0.97	1.99
Age×site (type)	8	4.35	3.04	6.78	0.64	0.73	7.87
Sex×site (type)	3	3.84	2.77	7.38	0.52	0.70	9.29
Age×sex×site (type)	3	6.85	29	2.49	2.75	0.06	3.13

[a] DF = degrees of freedom.
[b] MS = mean square.
[c] Pr = P-value.

correlated dependent variables, spleen weight, and PFCs using MANCOVA.

Before testing the main effect of contamination on spleen weight and PFCs, we determined, based on a three-level ANOVA, that age showed a significant interaction with type of site (contaminated or reference) for spleen weight (Table 2). However, there was no significant statistical interaction between age and PFCs (Table 3). Follow-up analysis showed no significant simple effects for the age by type interaction for spleen weight. In order to examine the effect of contamination irrespective of mouse age, we performed a MANCOVA on spleen weight and PFCs, treating age as a covariate. This analysis failed to reveal a significant multivariate effect for contamination exposure (Table 4). It is important to note that the age×type of site interaction was significant in the ANOVA, yet when age was accounted for as a covariate, type of site was nonsignificant. Therefore, differences between animals for spleen weight were due to age, but apparently not contamination. In addition, the ANOVA showed that variation across individuals (i.e., error variance)

TABLE 3. Results of the three-way analysis of variance (ANOVA) for number of plaque-forming cells.

Source	DF[a]	Type II MS[b]	Denominator DF[a]	Denominator MS[b]	*F*-value	Pr[c]>*F*	Percent of total variance
Age	4	0.95	7.16	0.70	1.37	0.33	2.12
Sex	1	0.002	3.09	0.80	0.0029	0.97	2.71
Type	1	1.38	5.41	0.51	2.69	0.16	5.96
Age×sex	2	0.26	2.61	0.84	0.31	0.765	6.29
Age×type of site[d]	3	0.23	8.28	0.70	0.33	0.80	0.26
Sex×type of site	1	0.50	1.98	0.79	0.63	0.51	3.25
Site (type)	5	0.42	0.58	0.66	0.64	0.77	3.97
Age×site (type)	7	0.69	2.33	0.84	0.82	0.64	0.73
Sex×site (type)	2	0.80	1.91	0.84	0.94	0.52	6.42
Age×sex×site (type)	2	0.84	25	0.84	1.01	0.38	11.06

[a] DF = degrees of freedom.
[b] MS = mean square.
[c] Pr = P-value.
[d] Type of site = contaminated or uncontaminated.

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TABLE 4. Results of the multivariate analysis of covariance (MANCOVA) performed on spleen weight and number of plaque-forming cells.

Parameter	Wilkes' λ	*F*-value	Numerator DF[a]	Denominator DF[a]	Pr[b]>*F*
Type of site[c]	0.94	1.70	2	52	0.19
Sex	0.97	0.89	2	52	0.42
Age	0.92	2.21	2	52	0.12

[a] DF = degrees of freedom.
[b] Pr = P-value.
[c] Type of site = contaminated or uncontaminated.

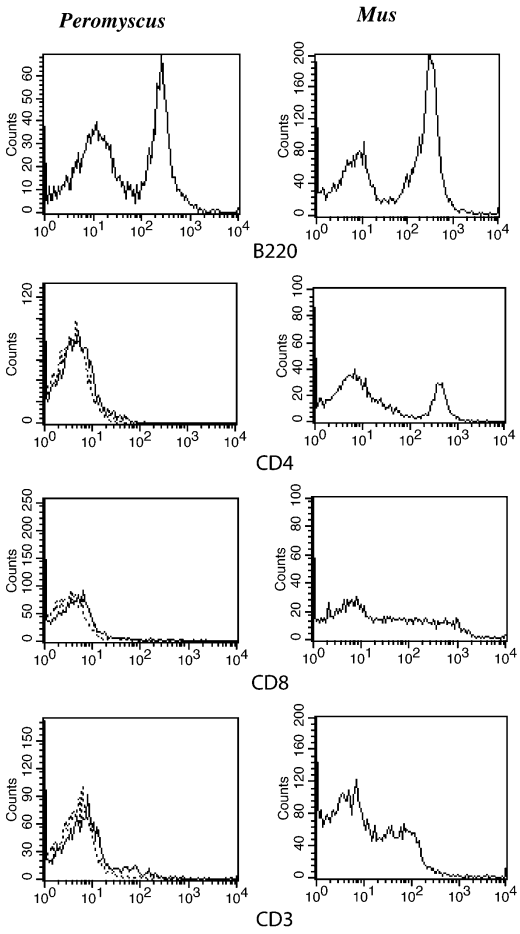


FIGURE 1. Cross-reactivity of *Mus*-derived antibodies with *Peromyscus* lymphocytes. Spleen cells from laboratory BALB/c mice or *Peromyscus* were stained with antibodies to *Mus* surface proteins including anti-CD3, anti-CD4, anti-CD8, and anti-B220. Dashed lines indicate isotype controls with the appropriate dye for each specific antibody. Figures show a representative animal for each genus.

comprised 57% of the overall variance for both parameters. Therefore, any differences in mouse spleen weight or PFCs due to contamination differences at the sites would have to be very large in order to be detected.

We determined percentages of B cells using the 6B2 monoclonal antibody against mouse B220 because there was sufficient cross-reactivity between the *Mus* epitope and its *Peromyscus* counterpart. Unfortunately, we did not observe any staining of T cells using monoclonal anti-mouse CD3, anti-CD4, or anti-CD8; hence we cannot report T-cell counts. Recently sequenced *Peromyscus* cDNAs for CD3 and CD4 mRNAs predicted that these proteins share only 66% and 76% amino acid identities, respectively, with those of *Mus* (M. Rangel and B. Hjelle, unpubl. data). An example of the flow cytometry staining for *Mus* and *Peromyscus* is shown in Figure 1. Variation in percent B cells was high, ranging from 21.38 to 80.31 for contaminated sites and ranging from 5.84 to 73.68 for the reference sites; this high variance across individuals contributed to the lack of significance between type of site (see below). We analyzed the contaminant effect on the proportion of B cells with a separate ANOVA because there was no significant correlation between percent B cells and either spleen weight or mean number of PFCs.

A three-way ANOVA of percent B cells showed no significant effect of age ($F_{4,6}$=0.81, P=0.56); therefore we pooled B cell counts of individual mice across all

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TABLE 5. Results of the two-way analysis of variance (ANOVA) for B cell number.

Source	DF[a]	Type III MS[b]	Denominator DF[a]	Denominator MS[b]	*F*-value	Pr[c]>*F*	Percent of total variance
Sex	1	0.01	47.37	0.37	0.03	0.87	2.69
Type of site	1	0.47	8.79	1.19	0.39	0.55	4.01
Sex×type	1	0.63	47.47	0.37	1.68	0.20	4.55
Site (type)	5	1.12	5	0.09	11.60	0.10	5.19
Sex×site (type)	5	0.09	50	1.55	0.06	0.99	10.78

[a] DF = degrees of freedom.
[b] MS = mean square.
[c] Pr = P-value.
[d] Type of site = contaminated or uncontaminated.

age classes. We analyzed the effect of contamination using a two-way ANOVA where sex and type of site were treated as fixed effects, and specific site location (site) was nested within type of site and treated as a random effect (Table 5). This analysis only revealed a significant effect of site, and potential exposure to contaminants explained little of the variance. However, the interindividual or error variance in this ANOVA comprised 73% of the variance, with site contributing only a very small amount to the total variance. It is therefore likely that the significant result of site as a factor was merely due to the large error term used to calculate the *F* statistic in the ANOVA.

DISCUSSION

Beland et al. (1993) found a high occurrence of ciliate protozoan pneumonia in Beluga whales (*Delphinapterus leucas*) from the St. Lawrence River, which was heavily contaminated with organochlorines. They suspected suppression of the immune system of these whales, but had no conclusive evidence because they did not perform assays for immunocompetence. Recent applications of sophisticated immunologic assays to wild animals in natural or seminatural environments, however, have provided such assessments of contaminants' impact on the immune system. For example, Trust et al. (1990) used penraised mallards (*Anas platyrhynchos*) to investigate the effects of lead ingestion on lymphocyte transformation responses to two mitogens and on antibody titers to SRBC. Similarly, Rocke and Samuel (1991) studied lymphocytes and antibody production to SRBCs in mallards that lived in enclosures with high lead shot density, and Propst et al. (1999) assessed the immunotoxicity risk to laboratory-raised cotton rats (*Sigmodon hispidus*) exposed in situ on a Superfund waste site in Oklahoma to soils contaminated with complex mixtures of organic hydrocarbons and heavy metals. Despite the fact that they found some immune alterations, they concluded that small mammals exposed as long-term residents may be better for biomonitoring than wild animals introduced as mesocosms. Grasman and his colleagues (2001, 2002) demonstrated successful application of numerous methods to screen wildlife for immunotoxic effects, especially fish-eating birds that are resident at PCB-contaminated Great Lakes (Grasman, 2002; Grasman and Fox, 2001). Although immunotoxicology studies provide essential data on altered immune responses for wildlife, they often do not provide estimates of potential and actual exposure to contaminants. The latter information is vital to establish a database that allows comparisons between laboratory and field studies and enhance our understanding of dose-response relationships between contaminant levels and target systems.

A major concern in the investigation of immunocompetence in natural populations is that wild-caught animals vary with

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respect to age, sex, and genetic background, which are factors that can affect immune responses beyond environmental factors (Nelson and Blom, 1994; Klein and Nelson, 1997; Pillet et al., 2000; Bunn et al., 2001; Prendergast et al., 2002). It is well known that the humoral immune response declines in quality and quantity as animals age (Miller, 1996). Attempts can be made to sample animals from a single sex and age class; however, accurate determination of age may be difficult in the field. Furthermore, variability in genetic backgrounds, and hence in the natural immune response of wild mammals, cannot be eliminated. Therefore, immune suppression of individual mice due to pollution will only be detectable if it is not exceeded by the natural variation in immune response of such wild animals.

We compared the immune response of carefully aged wild mice of both sexes and statistically accounted for contributions of intraindividual variation, age, and sex to immunocompetence. Our ANOVAs confirmed that individuals varied greatly with respect to their immune responses; individual variation explained between 57% and 73% of the overall variance for the three immunologic variables. This variation may have been the main reason why residence on contaminated sites had no statistical effect on any of the three immunologic parameters. Therefore, statistical demonstration of immunotoxicity in natural populations will require large sample sizes and large treatment effects.

We also demonstrated that accurate knowledge of mouse age is essential in immunotoxicology studies. Although age had no effect on either B cells or spleen PFCs, age affected spleen weight of our mice differently for contaminated and reference sites. However, the particular contaminant level at which age statistically affected spleen weight could not be identified because of the lack of significant simple statistical effects. We conclude that ecotoxicology field studies should minimize variation across individuals as much as possible, have large sample sizes, and avoid spleen weight as a measure of immunopathology if the age distribution of sampled individuals is expected to vary greatly.

Several laboratory studies have demonstrated sex-dependent immune responses to heavy metal exposure that may be indicative of a differential sensitivity to contaminants for males and females in the field (Pillet et al., 2000; Bunn et al., 2001; Kakela et al., 2002). At the same time, female mice tend to be philopatric (Hoffmeister, 1989), and if born into a contaminated habitat, they might be exposed to pollutants for a much longer period of time than males. We tested for immunopathology and humoral immunity for male and female mice at our contaminated and reference sites; however, males and females did not consistently differ in immunocompetence. Although we recorded the general reproductive condition of animals, such as being sexually immature or mature, or lactating or pregnant, our sample size did not allow the necessary subsampling to statistically separate reproductive condition and age and to discern if reproductive condition separately contributed to variance in immune response. In addition, this study was not designed to eliminate or statistically address variation in immune response due to additional uncontrolled variables, such as intraindividual variation in diet, physiologic stress, or hormone levels within age classes (Nelson and Demas, 1996; Kakela et al., 2002).

Lack of a relationship between potential contaminant exposure and immune response in our study might be because resident mice were not exposed to critical levels of mixtures of heavy metals. Unfortunately, threshold levels of exposure that impact immune systems in wild animals are not known. Nonetheless, many immunologic studies of laboratory animals have shown that doses of at least 1,000 ppm lead were required to elicit a significant decrease in immunocompetence (Luster et al., 1980; Neilan et al., 1980; Lawrence, 1981; Koller, 1990; McMurry et

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al., 1995). The highest values of lead obtained from soil and tissue samples in our study were less than half that amount, and hence actual exposure of at least this one heavy metal may have been below the threshold level. Thus, perhaps a blending of laboratory and field studies would be of use in the future, particularly for establishing contaminant thresholds that affect the immunologic assays used in this study. Also the use of mixtures and levels of heavy metals documented in our soil and tissue samples in a laboratory setting with inbred mice would eliminate the variable of genetic diversity yet provide a more realistic level of environmental exposure.

In addition to applying traditional assays to examine immunocompetence, we tested applicability of flow cytometry to quantify lymphocyte numbers in the white-footed mouse. Although we could label and quantify B cells using monoclonal antibodies, we failed to successfully bind antibodies to T cells despite considerable effort. Commercially available antibodies developed for laboratory mice may lack binding specificity for *Peromyscus* T cells (T. Schountz, pers. comm.), which would explain our inability to label them with such antibodies. Future studies using flow cytometry could provide information not only on enumeration of B cells, but their subset distribution and activation state, which might be altered by exposure to contaminants. However, each individual *Mus*-reactive reagent will need to be tested for cross-reactivity to *Peryomyscus* or a new set of reagents developed.

In summary, our study successfully utilized flow cytometry, spleen weight, and spleen plaque-forming assays as tools to evaluate the immunocompetence of wild mice. Based on these comprehensive assays, we did not detect any differences in immune response for mice collected at contaminated versus reference areas. This result is most likely a consequence of interindividual variation in immune response due to individual differences in genetic background, physiology, and response to chemicals. Alternatively, insufficient exposure of animals to these mixtures of heavy metals could have been responsible for the apparent lack of immunosuppression.

Based on our research, we encourage additional studies that provide estimates of bioavailability and bioaccumulation of pollutants in combination with assays of immunosuppression to establish a database on the potential risks of complex mixtures of environmental pollutants to mammalian wildlife. In addition, we recommend that immunotoxicologists establish the age and reproductive condition of each wild animal and focus their investigation on large samples of one sex to reduce variability and improve statistical power for their analysis.

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