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Acute Effects of Benzene and Cyclophosphamide Exposure on Cellular and Humoral Immunity of Cotton Rats, *Sigmodon hispidus*

Scott T. McMurry,[1] Robert L. Lochmiller,[1] Michelle R. Vestey,[1] Charles W. Qualls, Jr.,[2] and Chandikumar S. Elangbam[2]

[1]Department of Zoology and [2]Department of Veterinary Pathology, Oklahoma State University, Stillwater, Oklahoma 74078, USA

Many environmental pollutants are potent immunotoxicants with the capability of altering host resistance to pathogens and compromising overall immunological integrity (Vos 1977, Faith et al. 1980). Compounds known to be immunotoxic in the environment include halogenated biphenyls (PCB, PBB), polychlorinated dibenzo dioxins (TCDD), heavy metals (cadmium, lead), and several pesticides (DDT). Because environmental contaminants typically occur as a diverse group of compounds that are difficult to individually identify, their hazardous potential is often difficult to study in the laboratory (Rowley et al. 1983). Consequently, development of wild mammalian species as bioindicators of environmental contamination may provide an alternative approach to assessing the hazardous potential of immunotoxicants for both human and wildlife populations.

The cotton rat (*Sigmodon hispidus*) is an excellent prospect as an *in situ* bioindicator of environmental contamination of terrestrial ecosystems (Elangbam et al. 1989). They are phylogenetically closely related to man, are in close contact with soil and vegetation (the liklihood of contaminant exposure is high), and have small home ranges (less than 1 hectare). In addition, cotton rats are ubiquitously distributed throughout the southeastern United States, easily captured, and have a short generation interval (less than 1 year), making them more suitable as a potential bioindicator than larger wild mammals.

Recently, we initiated studies in our laboratory to develop a sensitive method to evaluate environmental immunotoxicity hazards to wildlife and humans. The present study was designed to test the sensitivity of selected humoral and cell-mediated immune response assays in the cotton rat to acute benzene and cyclophosphamide exposure which are known immunotoxicants (Dean et al. 1979, Snyder 1987).

MATERIALS AND METHODS

A total of 72 adult cotton rats (mean weight = 103g$\pm$3.7(SE)) was collected between September 19th and November 9, 1989 from tallgrass prairie habitat approximately 1 km west of Stillwater, Oklahoma. Cotton rats were returned to the laboratory animal facility and housed singly or paired in polystyrene cages with wood chip bedding. Animals were provided water and food

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Send reprint requests to S. T. McMurry at the above address.

(Purina Rodent Chow) ad libitum under natural light:dark conditions during a 12-day acclimation and 11-day experimental period.

Cotton rats were randomly assigned to 1 of 6 experimental treatments, with 12 animals per treatment. Treatments included 4 benzene (Aldrich Chemicals, Milwaukee, WS) dosages (100, 300, 600, or 1000 mg/kg body weight), cyclophosphamide (CY, Sigma Chem. Co., St. Louis, MO) as a positive control (50 mg/kg body weight), and corn oil as a negative control. All treatments were administered intraperitoneally (IP) for 3 consecutive days. Benzene was administered in corn oil on days 1-3 of the experimental period. Positive and negative controls received corn oil on days 1-3 at volumes equal to benzene doses. Cyclophosphamide in distilled water was administered to positive control animals on days 8-10. On day 7, all cotton rats received a 0.2 ml IP injection of a 10% sheep red blood cell (SRBC, Colorado Serum Co., Denver, CO) suspension in PBS. The trial was terminated and necropsies performed on day 12 and weights recorded for thymus gland, liver, spleen, and paired kidneys and adrenal glands.

Animals were anesthetized with an intramuscular injection of Ketamine hydrochloride (Bristol Laboratories, Syracuse, NY) at 50 mg/kg body weight. Blood was collected, via the retro-orbital sinus plexus, into 3-ml vacutainers (Becton Dickinson, Rutherford, NJ) containing EDTA (K_3). Hematocrits were determined by the microcapillary-centrifuge method. White blood cell (WBC) counts were determined manually using a hemacytometer. Whole blood smears were prepared for each animal and differential leukocyte counts performed on 100 cells.

Animals were sacrificed by cervical dislocation while under ketamine anesthesia. Spleens were aseptically removed, placed in preweighed 15x60 mm sterile petri dishes containing supplemented RPMI 1640 medium (RPMI-S, Sigma Chem. Co., St. Louis, MO) and weighed to the nearest 0.1 mg. RPMI-S consisted of 100 ml of RPMI 1640 medium containing 1.025 ml L-glutamine (200mM, Sigma), 1.0 ml Na pyruvate (100mM, Sigma), 1.0 ml non-essential amino acids (10mM, 100x, Sigma), 1.0 ml penicillin (10,000 U/ml) /streptomycin (10 mg/ml, Sigma), 0.1 ml 2-mercaptoethanol ($2x10^{-2}$ M, 1:1000 dilution in sterile PBS, Sigma), and 11.5 ml normal horse serum (Sigma). Spleens were cut into 3-4 pieces and gently disrupted in a sterile glass-on-glass tissue homogenizer (0.15 mm clearance) containing 5 ml ice-cold RPMI-S. Cells were allowed to settle for 10 min. and supernatant decanted into sterile 16x125mm screw-cap culture tubes. Cells were centrifuged for 7 min. at 10 C and 275xg, supernatant decanted, and pellet resuspended in 5 ml of RPMI-S (this wash step was performed 3 times). Viable cell counts were performed with a hemacytometer after lysing erythrocytes in Tris-buffered 0.83% ammonium chloride (Tris/Cl, Sigma) and staining with Trypan blue (Sigma).

Lymphocyte proliferation, after *in vitro* mitogenic stimulation with Concanavalin A (Con A) and Pokeweed mitogen (PWM), was assessed by cellular reduction of tetrazolium salt (Mosmann 1983). Spleen cell suspension was adjusted to a final concentration of 500,000 cells/90 ul in RPMI-S. Four concentrations of each mitogen were added (10 ul volumes) to duplicate aliquots (90 ul) of the final cell suspension in 96 well, flat bottom microtitre plates. Five concentrations of each mitogen (ug/ml of culture) were used: Con A (0, 2.5, 5, 10, and 20) and PWM (0, 0.156, 0.313, 0.625, and

1.25). Cells were incubated for 72 hr at 37°C in a humidified incubator (5% CO^2). After 69 hr, 10 ul of MTT (tetrazolium salt, 5 mg/ml in PBS, Sigma) were added to each well, incubated the remaining 3 hr, and 160 ul of acid-isopropanol (176 ul concentrate HCl in 49.824 ml isopropanol) were added to each well. Absorbances at 570nm/630nm were recorded on a Titertek Multiskan Plus MK II (Flow Laboratories, McLean, VA) against unstimulated controls as blanks.

A modification of the technique used by Cunningham and Szenberg (1968) was used to assess humoral immunity mediated by IgM secreting splenocytes. Spleen cell preparations from each animal were centrifuged as previously described and resuspended in 5 ml Tris/Cl. Cell suspension was then underlayed with 1 ml of fetal bovine serum (Sigma Chem. Co., St. Louis, MO), allowed to stand for 7 minutes, then centrifuged. The pellet was washed twice in 5 ml of ice-cold PBS containing 5% fetal bovine serum (PBS-F). Cell counts were performed and final cell concentration adjusted to $9.6x10^6$ cells/ml. A reaction mixture was prepared consisting of either 200 ul of cell suspension plus 20 ul PBS-F or 50 ul cell suspension plus 170 ul PBS-F. To each mixture were added 80 ul of 25% SRBC in PBS and 100 ul of 25% guinea pig serum (Bioproducts for Science, Inc.) in PBS resulting in a final volume of 400 ul. Cunningham chambers prepared from glass microscope slides were loaded with 125 ul of each reaction mixture, sealed with a 1:1 vaseline/paraffin mixture, and incubated at 37°C for 2 hr. Plaques were enumerated using a dissecting microscope.

Delayed-type hypersensitivity responsiveness (DTH) was measured in cotton rats sensitized on day 4 with a percutaneous application of 100 ul of 3% 4-Ethoxy-methylene-2-phenyl-oxazol-5-one (oxazolone, Sigma) in absolute alcohol to shaved abdomens. Animals were challenged on day 11 with 50 ul of 3% oxazolone and absolute alcohol to the left and right ear, respectively. Immediately after sacrifice (24 hr post-challenge), both ears were removed and weighed to the nearest 0.1 mg. DTH was measured as percent weight change of the challenged (left) ear relative to the control (right).

Statistical differences between treatment and control groups were determined at the 10% level of significance. Differences in organ morphology and immune responsiveness were determined by one-way analysis of variance (SAS 1985). Duncans multiple range test was used for comparisons among treatment means when main effects were different. Specific contrasts (single degree of freedom) were used in all analysis of variance procedures to compare differences between major treatment components (benzene vs. negative control, benzene vs. positive control, negative vs. positive control, and 100mg benzene vs. 1000mg benzene).

RESULTS AND DISCUSSION

There were no gross behavioral changes in any of the cotton rats receiving benzene. Two animals, one each from negative and positive control groups, died during the experiment from non-treatment related causes. Wierda et al. (1981) noted that C57BL/6 mice exposed to 440-660 mg/kg benzene exhibited piloerection, excitation, tremors as well as some mortality. Although dosages of 1000 mg/kg of benzene were administered to cotton rats we observed no neuromuscular abnormalities or mortality.

Thymus weights were markedly depressed among CY-treated cotton rats compared to benzene-treated ($P < 0.003$) and negative control ($P < 0.02$) groups (Fig. 1). A dose response ($P < 0.006$) in spleen weights was indicated among benzene treatments, with greater spleen weights among cotton rats receiving 1000 mg/kg than 100 mg/kg. Cyclophosphamide-treated animals also had significantly ($P < 0.05$) lower spleen weights than the 1000 mg/kg benzene group. Mean weights of liver and paired kidneys and adrenal glands did not differ ($P > 0.10$) among treatment groups.

Hematocrits, WBC counts and viable splenocyte yields were significantly ($P < 0.05$) influenced by experimental treatments (Fig. 2). WBC counts (cells x $10^3/mm^3$) were greatest for cotton rats receiving 600 mg/kg benzene ($\bar{x} \pm SE = 7.99 \pm 1.37$) and lowest for CY-treated animals (3.45 ± 0.55). Total viable splenocyte yields (cells x 10^6) were greatest among animals receiving 1000 mg/kg benzene (151.5 ± 19.1) and lowest among those exposed to CY (70.1 ± 8.6).

Animals treated with CY showed significant depressions in both WBC counts and viable splenocyte yields compared to benzene-treated ($P < 0.005$) and negative control ($P < 0.04$) groups. Hematocrits also showed a significant ($P<0.04$) depression in CY-treated compared to benzene-treated cotton rats. WBC counts showed an elevated dose response ($P<0.10$) between 300 and 600 mg/kg, but levels were not significantly different ($P > 0.10$) from negative controls. Similarly, viable splenocyte counts demonstrated an elevated dose response ($P<0.05$) between cotton rats receiving either 100 or 300 mg/kg benzene and those dosed with 1000 mg/kg benzene, but levels were not significantly different ($P > 0.10$) from negative controls.

Absolute counts of blood leukocyte subpopulations indicated that only lymphocytes exhibited a significant ($P < 0.001$) response to experimental treatments (Fig. 2). Cotton rats treated with CY had significantly ($P < 0.001$) lower lymphocyte counts than benzene treatments and negative controls. Mean neutrophil counts (cells x $10^3/mm^3$) ranged from 2.12 ± 0.33 for cotton rats receiving 1000 mg/kg benzene to 3.33 ± 0.92 for those given 100 mg/kg. Numbers of eosinophils (0.09 ± 0.01), basophils (0.04 ± 0.01), and monocytes (0.02 ± 0.01) exhibited considerable variation and were not influenced ($P > 0.10$) by experimental treatments.

Total number of SRBC-responding plaque forming cells per spleen (PFC/spleen) and per unit weight of spleen (PFC/mg spleen) differed ($P < 0.05$) significantly across experimental treatments (Fig. 3). Cotton rats receiving either 300 mg/kg or 1000 mg/kg benzene had more PFC/spleen ($P < 0.05$, $P < 0.03$) and PFC/mg spleen ($P < 0.05$, $P < 0.06$, respectively) than those in the 100 mg/kg treatment group. Specific contrasts indicated that both PFC/ spleen ($P < 0.04$) and PFC/mg spleen ($P < 0.07$) were greater for benzene-treated than CY-treated cotton rats. Differences between negative control animals and other treatment groups were not significant ($P > 0.10$).

The highest number of PFC/10^6 cells observed for any cotton rat in this experiment was 58.8 in an individual receiving 300 mg/kg benzene. Mean PFC/10^6 cells ranged from 2.22 ± 0.88 for cotton rats receiving 100 mg/kg benzene to 13.06 ± 4.89 for the 300 mg/kg benzene group (Fig. 3). Significant differences in number of PFC/10^6 cells among experimental treatment groups were limited to greater ($P < 0.06$) responses in the 300 mg/kg benzene group

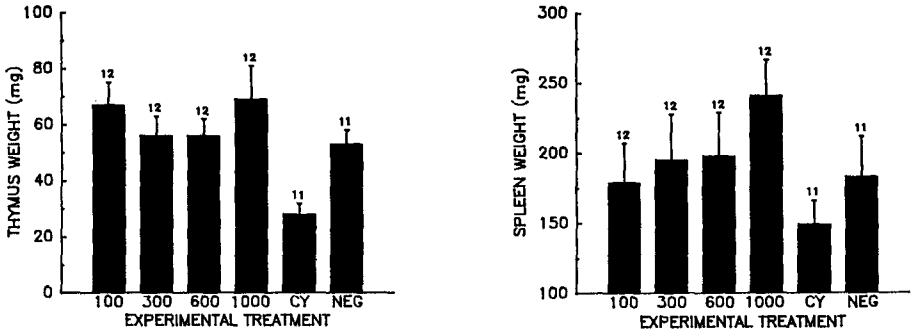

Figure 1. Differences in mean thymus and spleen weights (±SE) from adult cotton rats dosed intraperitoneally with benzene (100, 300, 600, and 1000 mg/kg body weight), cyclophosphamide (CY, 50 mg/kg body weight), and corn oil (NEG) for 3 consecutive days. Values above bars represent sample size (n).

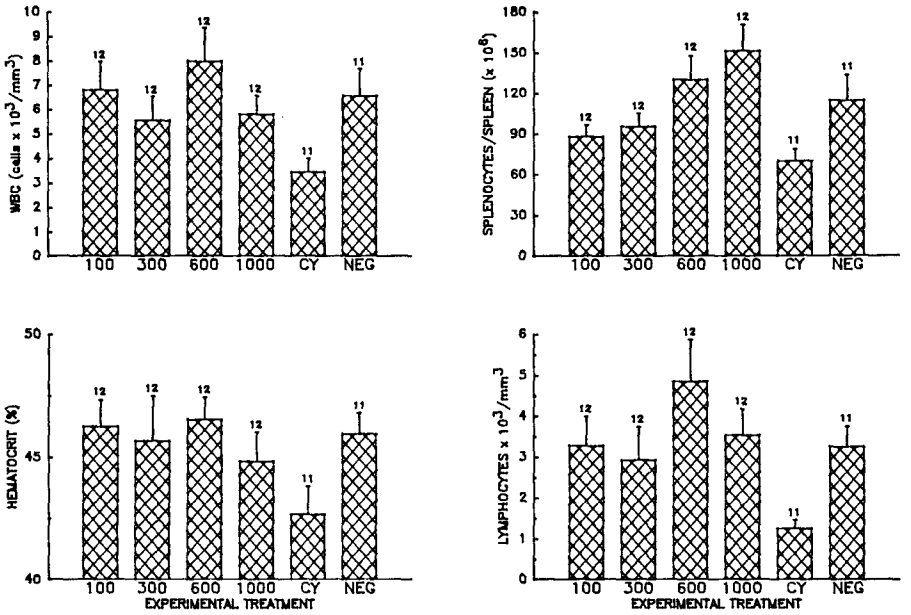

Figure 2. Differences in mean white blood cell counts (WBC), viable splenocytes/spleen, hematocrits, and total lymphocytes (±SE) from adult cotton rats dosed intraperitoneally with benzene (100, 300, 600, and 1000 mg/kg body weight), cyclophosphamide (CY, 50 mg/kg body weight), and corn oil (NEG) for 3 consecutive days. Values above bars represent sample size (n).

than 100 mg/kg benzene and CY groups.

Optimum doses for Con A and PWM stimulation were 5 and 0.625 ug/ml

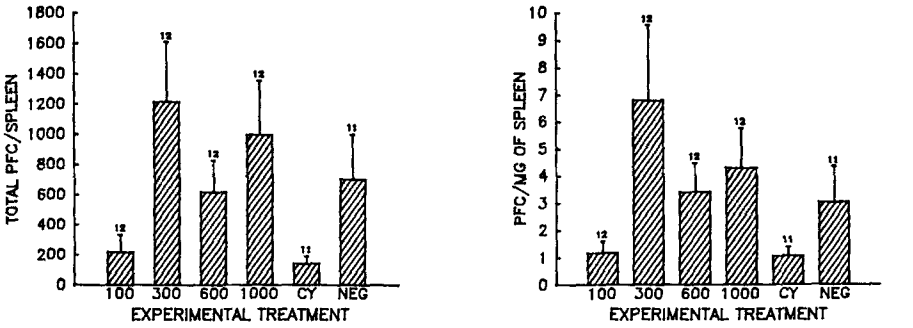

Figure 3. Differences in mean number of plaque forming cells (PFC) per spleen, per mg of spleen, and per 10^6 splenocytes (±SE) from adult cotton rats dosed intraperitoneally with benzene (100, 300, 600, and 1000 mg/kg body weight), cyclophosphamide (CY, 50 mg/kg body weight), and corn oil (NEG) for 3 consecutive days. Values above bars represent sample size (n).

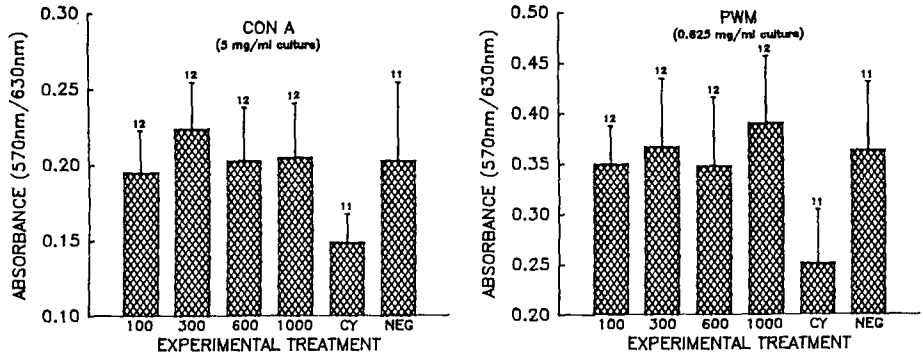

Figure 4. Differences in mean absorbance values (±SE) of splenocytes stimulated with Concanavalin A (Con A) and Pokeweed mitogen (PWM) from adult cotton rats dosed intraperitoneally with benzene (100, 300, 600, and 1000 mg/kg body weight), cyclophosphamide (CY, 50 mg/kg body weight), and corn oil (NEG) for 3 consecutive days. Values above bars represent sample size (n).

culture, respectively. No significant differences ($P > 0.10$) among treatments were observed in lymphoproliferative responsiveness to either Con A or PWM (Fig. 4). Mean absorbances of cultures stimulated with Con A ranged from 0.149±0.02 for the CY-treated group to 0.224±0.03 for those receiving 300 mg/kg benzene. Mean absorbances for PWM-stimulated cultures ranged from 0.251±0.05 for cotton rats exposed to CY to 0.390±0.07 for those receiving 1000 mg/kg benzene. Specific contrasts indicated that mean

lymphoproliferative responses to PWM for benzene-treated ($P < 0.07$) and negative control ($P < 0.10$) animals were higher than for CY-treated animals. Mean absorbances of nonstimulated cultures were significantly greater among CY-treated cotton rats than for benzene-treated ($P < 0.05$) or negative control ($P < 0.02$) animals.

Cellular immunity measured as immune responsiveness to a topically applied antigen showed no significant ($P > 0.10$) differences among experimental treatments. DTH responses ranged from $18.6 \pm 3.2\%$ for cotton rats receiving 300 mg/kg benzene to $28.4 \pm 5.0\%$ for those treated with 1000 mg/kg. Overall, mean DTH response of pooled benzene- and CY-treated animals was $23.4 \pm 2.1\%$ which was only slightly lower than negative controls ($26.5 \pm 5.6\%$).

Benzene is highly toxic to bone marrow stem cells (Snyder and Kocsis 1975), mitotic cells (Parmentier and Dustin 1948), and lymphocytes, especially B cells and suppressor T cells (Irons et al. 1979, Aoyama 1986, Snyder 1987), appear to be especially sensitive to benzene exposure. Functional impairment of cell-mediated immunity has also been documented in mice (Rosenthal and Snyder 1987), which tend to be more susceptible to a given benzene dose than rats (Snyder et al. 1978). Wierda et al. (1981) demonstrated a significant acute benzene-induced (44 to 660 mg/kg) depression of spleen and blood cellularity, lymphoproliferative ability, and IgM production in C57BL/6 mice. However, benzene and benzene metabolites are not always suppressive and at low doses (Pfeifer and Irons 1981) or on cellular immunity (Aoyama 1986) can be stimulatory.

Although some apparent dose related responses to benzene were seen (PFC/mg spleen, PFC/spleen, spleen weight), acute benzene exposure induced neither immunosuppression or stimulation in cotton rats, given the exposure regime used in our study. Previously, many laboratory animal studies examining the immunotoxic effects of benzene exposure typically sacrificed after the last dosage of benzene was administered (Weirda et al. 1981, Rosenthal and Snyder 1985, Aoyama 1986, Rosenthal and Snyder 1987). Conversely, our cotton rats were sacrificed 9 days after the last of 3 consecutive benzene dosages to duplicate the time-lag factors associated with using *in situ* biomonitors which have to be transported from the field (site of exposure) to the laboratory before immunoassays are performed. This difference in dose regime might explain the apparent discrepancies that exist among studies. Benzene is readily metabolized in rodents (Snyder and Kocsis 1975) and dosages of 880 mg/kg may be completely metabolized in mice within 24 hr of exposure (Gerarde and Ahlstrom 1966). Mice are capable of expiring 70% of an 880 mg/kg dosage of benzene within 8 hr after administration (Snyder and Kocsis 1975). Rosenthal and Snyder (1985) examined levels of *Listeria monocytogenes* infection in C57B1/6J mice during benzene exposure and found mice capable of recuperating from all but the highest doses (300 ppm) 4 days after exposure. Induction of the mixed function oxidase (MFO) enzyme system in cotton rats exposed to xenobiotics has been documented (Elangbam et al. 1989). However, differences between rate of biotransformation in cotton rats and laboratory mice is unknown.

Two distinct pathways of lymphopoiesis involving either 2-3 or 6-8 mitotic divisions, have been identified in rodents (Leblond and Sainte-Marie 1960, Osmond and Everett 1964). The short pathway occurs in bone marrow and

turnover times average 3 days or less; the duration of the long pathway in lymphoid tissue is unclear. Our data strongly suggests that metabolism and excretion of metabolites in the cotton rat were probably complete by 9 days after benzene exposure. Up to 80% of the pluripotent hematopoietic stem cells may be quiescent under normal conditions but may be induced to proliferate in response to benzene-induced depression of more differiented cells (Snyder 1987).

Cyclophosphamide, like benzene, is quickly metabolized and targets mitotic cells (Bach and Strom 1985) and its effectiveness as an immunosuppressant is lost within a few days after challenge (Shand 1979). Cyclophosphamide has been shown to suppress antibody production and lymphocyte proliferation in mice (Dean et al. 1979) and enhance DTH responsiveness through selective elimination of suppressor T cells (Shand 1979). Cyclophosphamide administered to cotton rats 3 times per week at 50 mg/kg has been reported to be immunosuppressive (Johnson et al. 1982). Cyclophosphamide was administered 2 days prior to sacrifice in this study and immunosuppression was indicated by decreased WBC and lymphocyte counts, splenocyte yields, spleen weight, and lymphoproliferative response to PWM. Discrepancies between immunosuppressive activity of CY and benzene probably result from differences in the protocol.

Variability in immunological responsiveness among cotton rats, presumably of genetic origin (Biozzi et al. 1984) and possible variation among individuals to benzene toxicity, was observed in this study. The lack of benzene-induced immunosuppresion in this study suggests that cotton rats have efficient biotransformation capabilities and can apparently recuperate quickly from leukopenia, thus highlighting necessary precautions when using *in situ* biomonitors of immunotoxicity. Animals caught on contaminated sites and held in the laboratory during acclimation and/or immunization periods may eliminate considerable amounts of xenobiotics and recover from abnormalities caused by the contaminants. However, we still maintain the feasibility of the cotton rat as an in situ biomonitor based on the positive findings with CY-induced immunosuppression.

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