

# Endocrine Disruption in Adolescence: Immunologic, Hematologic, and Bone Effects in Monkeys

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Environmental contaminants with estrogenic properties have the potential to alter pubertal development. In addition to the reproductive system, other systems that mature under the influence of estrogen could be affected. This study examined the effect on immune, hematologic, and bone mass parameters of treatment with estrogenic agents (methoxychlor, MXC, 25 and 50 mg/kg/day; diethylstilbestrol, DES, 0.5 mg/kg/day) given in the peripubertal period to female rhesus monkeys. DES had striking effects on several parameters assessed measures CBC and clinical chemistry including hematocrit, hemoglobin, serum albumin, liver transaminases, and lipids. Circulating lymphocytes, particularly B cells, were depressed by DES, and a maturational shift in a memory T-cell population was altered. Bone mass and length, as measured after a 9-month recovery period, were significantly lower in the DES group and bone mass tended to be reduced in the femur of the MXC50 group relative to controls. In conclusion, the data indicate that DES had a clear effect on immunohematology and bone growth, while MXC influenced fewer parameters. Disruption in these systems during puberty could alter adolescent risk for anemia and infectious disease and subsequent adult risk for diseases such as osteoporosis, heart disease, and autoimmune disease.

**Key Words:** endocrine disruption; rhesus monkeys; methoxychlor; diethylstilbestrol; hematology; flow cytometry; bone mineral density; puberty; female.

Endocrine disruption research seeks to understand the cellular actions and public health consequences of exposure to a variety of chemicals that interact with the endocrine system. Part of this research involves screening chemicals for endocrine actions in *in vitro* and simple *in vivo* assays (Gray, 1998; O'Connor *et al.*, 2002). The screening research includes assays with pubertal exposures and endpoints in rats (Goldman *et al.*, 2000; Marty *et al.*, 1999; Stoker *et al.*, 2000). However, to identify potential health impacts, more intensive research is needed in appropriate animal models. Our focus is on the impact of exogenous estrogens on the maturation that occurs under the influence

of endogenous estrogens during puberty and adolescence in nonhuman primates.

Many organ systems besides the reproductive tract mature and become regulated by gonadal hormones at puberty and can be anticipated to be influenced by endocrine-disrupting chemicals. Among the systems known to be sex differentiated, to contain abundant estrogen receptors, and to show dramatic changes at the time of puberty are the immuno-hematological and skeletal systems. In the immune system, puberty is a time of change in thymic processing of T-cell populations and of establishment of reproductive tract mucosal immunity. In addition, at puberty, sex-differentiated red blood cell characteristics occur as the erythron expands to accommodate the adolescent growth spurt and differentiates in response to changes in body composition and menstrual blood loss (Beard, 2000; Bergstrom *et al.*, 1995; Soekarjo *et al.*, 2001). Puberty is also a time for intense bone mineralization accompanying long bone growth prior to epiphyseal closure (Bonjour *et al.*, 1991; Gilsanz *et al.*, 1991; Gordon *et al.*, 1991; Theintz *et al.*, 1992).

This paper reports data from hematology, clinical chemistry, flow cytometry and bone mineral density assays conducted during the peripubertal period in female monkeys treated with exogenous estrogens diethylstilbestrol (DES) and methoxychlor (MXC). These two agents are model endocrine disruptors that have been studied in a variety of *in vivo* or *in vitro* studies, including rodent puberty assays. Previous reports have described effects on growth, the reproductive system (Golub *et al.*, 2003), and behavior (Golub *et al.*, 2004) in this cohort of estrogen-treated pubertal monkeys. Puberty and adolescence are the final stages of development and are particularly prolonged and complex in primate species. By using these agents in a nonhuman primate model that closely resembles humans during this developmental period, actions that are particularly relevant to human health are addressed.

## MATERIALS AND METHODS

**Design/treatments.** Female rhesus monkeys 24 to 36 months of age were administered DES (0.5 mg/kg/day), methoxychlor (MXC 25 or 50 mg/kg/day), or vehicle control ( $n = 8$  per group). The agents were mixed into flavored

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commercial baby foods and administered with a feeding syringe at the same time each day (Golub *et al.*, 2003). Dose selection was discussed previously (Golub *et al.*, 2003). The 0.5 mg/kg/day dose given to pregnant rhesus monkey (Hendrickx *et al.*, 1979) was effective in producing a similar syndrome in offspring as has been seen in DES daughters. The 25 and 50 mg/kg MXC doses are the lowest effective doses in rodent studies (Gray *et al.*, 1989); human exposure-effect data is not available for MXC. The treatments resulted in a 40–70% increase in estrogenicity of blood as determined with an *in vitro* ERA activation assay. The treatment period encompassed the peripubertal period from 6 months before to 6 months after the average age of menarche (30 months of age in our colony). Full reproductive maturity and first pregnancy are typically seen at 42 months of age.

**Animal housing and care.** Monkeys born and raised in outdoor group caging at the California National Primate Research Center (CNPRC) were screened for health status and moved to indoor caging rooms 4 months prior to initiation of dosing. They were pair-housed in 120 × 65 × 79 cm cages (48 animals per room) with light (lights on 0600 h to 1800 h) and temperature (68–78°F) regulation. A partition allowed separation of pairs for food intake determinations and behavioral testing while permitting socialization at all other times. The Enrichment Coordinator at the CNPRC determined compatible pairs based on prior cage location and individual temperament.

Purina monkey diet (Lab Diet #5047, PMI Nutrition International, Richmond, IN) was provided twice daily at 0700 and 1500 h. The diet contained 220 ± 26 ppm genistein and 217 ± 21 ppm daidzein measured as the aglycone form of the isoflavone. Monkeys also received supplementary foods screened for plant estrogen content as enrichment. An automatic system with a spigot in each cage provided drinking water. Monkeys were observed each morning for health signs and referred to the veterinarians for treatment, if needed.

**Assurance of compliance with animal codes.** All protocols were approved prior to use by the University of California, Davis Animal Use and Care Administrative Advisory Committee and followed the requirements of the Animal Welfare Act and the Guidelines for the Use and Care of Laboratory Animals. Monkeys were housed at the CNPRC, an AAALAC accredited vivarium.

**Assays.** Regular blood sampling time points were at 3-month intervals from the initiation of treatment continuing through recovery. At each time point, blood samples were obtained for complete blood count (CBC), clinical chemistry panel, and flow cytometry. (A baseline (pre-treatment) sample was also obtained for CBC and flow cytometry.) These tests have been modified and standardized for rhesus monkeys at the clinical laboratories of CNPRC. In addition, an *in vitro* assay for natural killer (NK) cell activity (chromium release assay) (ref) was performed at the end of the treatment period by the immunology core laboratory at CNPRC, and a scan for bone mineral content and density was obtained after the recovery period using the dual-energy x-ray absorption (DEXA) method.

CBCs were performed by experienced technicians in the CNPRC Clinical Laboratories. The clinical chemistry 20 panel was performed at the UCD Veterinary Medical Teaching Hospital Clinical Chemistry department using a Hitachi 717 automated analyzer with standard quality assurance procedures.

Flow cytometry was performed with FACS Vantage (Becton Dickinson) instrument by CNPRC clinical laboratory personnel who had experience with each of the selected markers in rhesus monkey populations. Lymphocytes were gated from PBMC with side- and forward-scatter plots. BD anti-human antibodies were primarily used; CD45 and CD29 reagents were from Beckman-Coulter. Support for the validity of the markers for monkeys has been established through use at CNPRC and some specific investigations in the literature (Jacobsen *et al.*, 1993; Lafont *et al.*, 2000; Rappocciolo *et al.*, 1992; Reimann *et al.*, 1994)

DEXA scans utilized an ECLIPSE DXA System located at CNPRC with settings optimized for rhesus monkeys (Register *et al.*, 1997) using the Norland X-ray Bone Densitometer Operator's Guide. Areas scanned were femoral neck, global proximal femur, distal radius and ulna, lumbar vertebrae 2–4, and whole body.

NK-cell activity was assessed by experienced technicians in the Immunology Core Laboratories of CNPRC using a chromium release assay (Djeu, 1995). Isolated peripheral blood mononuclear cells (PBMCs) were used as effector

cells with both NK-sensitive (K562) and NK-resistant (P815) target cells. Target cells were loaded with chromium at 50 µCi per 10<sup>6</sup> cells. They were incubated for 4 h at 37°C in 96-well plates at target:effector ratios of 3:1, 11:1, 33:1 and 100:1. Additional target cells were incubated without effector cells to determine spontaneous chromium release. The supernatant was removed and counted overnight.

Peripheral cytokines (interleukin (IL)-4, IL-10) were measured in serum samples using commercial kits (Bionor AS).

**Statistical analysis.** All parameters were examined by analysis of variance (ANOVA) with Fisher's post-hoc test at each time point. Categorical variables were examined with chi-square or Fisher exact test. Statistical analysis utilized SAS or Statview software.

## RESULTS

### *Hematology: Red Blood Cells*

Values obtained at the end of treatment are presented in Table 1. Time course data for selected parameters are shown in Figure 1. Hematocrit was depressed during the treatment period for the DES group, with a corresponding lowering of the RBC numbers and hemoglobin values relative to controls (Table 1, Fig. 1). After discontinuation of treatment, these values normalized to control levels except that mean corpuscular hemoglobin (MCH) was higher in controls than the three treated groups (data not shown). Mean corpuscular value (MCV) appeared somewhat larger in the control group than in the treated groups (Table 1), but this trend was present at baseline and may have been responsible for the higher MCH values in the treated groups during the recovery period. MCHC did not differ from controls, indicating normal characteristics of the red blood cells.

### *Clinical Chemistry Parameters*

Values obtained at the end of treatment are presented in Table 2. Time course data for selected parameters are shown in Figure 1. Lower serum albumin concentrations in the DES group were a striking feature of the clinical chemistry panel (Table 2, Fig. 1). Total protein was also lower in the DES group than in controls, an effect attributable to the marked decrease in circulating albumin. The reduced albumin and total protein in the DES group's chemistry panel corresponded with the lower plasma protein seen in the CBC (Table 1).

Exogenous estrogen effects on fibrinogen, a parameter known to be increased by therapeutic estrogens, were not clear in this study. ANOVAs showed transient statistically significant increases in the DES group serum fibrinogen 3 and 6 months after initiation of treatment (data not shown), although there were no group differences at the end of treatment. Fibrinogen values, reported at 100 mg/dl intervals, ranged between 100 and 400 mg/dl in all animals throughout the study. Values greater than or equal to 300 mg/dl occurred only in the treated groups during the treatment period. However, three of the treated monkeys also showed fibrinogen concentrations ≥ 300 mg/dl at baseline.

Circulating levels of several hepatic enzymes used as markers of liver damage were influenced by DES treatment (Table 2). This included gradually increasing concentrations

TABLE 1  
Mean Hematology Values at the End of Treatment

	Group			
	Control	MXC 25 mg/kg/day	MXC 50 mg/kg/day	DES 0.5 mg/kg/day
WBC ( $\times 10^3/\mu\text{l}$ )	15.0 $\pm$ 0.6*	13.3 $\pm$ 0.9	12.7 $\pm$ 0.9	10.0 $\pm$ 1.1 <sup>c</sup>
RBC ( $\times 10^6/\mu\text{l}$ )	5.42 $\pm$ 0.09	5.55 $\pm$ 0.20	5.61 $\pm$ 0.14	5.07 $\pm$ 0.18
Hemoglobin (gm/dl)	13.5 $\pm$ 0.2	12.9 $\pm$ 0.3	13.4 $\pm$ 0.3	12.0 $\pm$ 0.3 <sup>c</sup>
Hematocrit (%)	40.7 $\pm$ 0.7	39.8 $\pm$ 1.2	40.7 $\pm$ 0.9	36.5 $\pm$ 1.0 <sup>b</sup>
MCV (fl)	75 $\pm$ 0.9	72 $\pm$ 0.7 <sup>b</sup>	73 $\pm$ 0.8 <sup>a</sup>	72 $\pm$ 1.0 <sup>a</sup>
MCH (pg)	24.9 $\pm$ 0.4	23.4 $\pm$ 0.4 <sup>b</sup>	23.9 $\pm$ 0.2	23.7 $\pm$ 0.4 <sup>a</sup>
MCHC (pg/fl)	33.2 $\pm$ 0.3	32.5 $\pm$ 0.4	32.9 $\pm$ 0.3	32.8 $\pm$ 0.2
Platelets ( $\times 10^5/\mu\text{l}$ )	4.78 $\pm$ 0.34	4.61 $\pm$ 0.25	5.02 $\pm$ 0.25	3.40 $\pm$ 0.39 <sup>b</sup>
Plasma protein (gm/dl)	7.1 $\pm$ 0.1	7.2 $\pm$ 0.2	7.2 $\pm$ 0.2	6.9 $\pm$ 0.1
Fibrinogen (mg/dl)	175 $\pm$ 16	188 $\pm$ 23	138 $\pm$ 18	200 $\pm$ 27
Seg. Neutrophils ( $\mu\text{l}$ )	6453 $\pm$ 767	5178 $\pm$ 690	5704 $\pm$ 804	5089 $\pm$ 1160
Lymphocytes ( $\mu\text{l}$ )	7802 $\pm$ 622	7612 $\pm$ 492	6661 $\pm$ 648	4647 $\pm$ 547 <sup>c</sup>

\*Mean  $\pm$  SEM,  $n = 8/\text{group}$ .

<sup>a</sup>Significantly different from control, post-hoc  $t$ -test ( $p \leq 0.05$ ).

<sup>b</sup>Significantly different from control, post-hoc  $t$ -test ( $p \leq 0.01$ ).

<sup>c</sup>Significantly different from control, post-hoc  $t$ -test ( $p \leq 0.001$ ).

of transaminases (alanine amino transferase (ALT), aspartate amino transferase (AST)) to levels higher than controls by the end of treatment (Fig. 1). Gamma glutamyl transferase was at first depressed, rose to levels higher than controls 3 months after discontinuation of treatment, then returned to control levels (data not shown). Bilirubin was also slightly elevated in the DES and MXC25 groups at the end of treatment.

Serum lipids demonstrated an estrogenic effect, a rise in cholesterol and triglycerides, in the DES group. The effect on triglycerides was also significant in the MXC25 group at the end of treatment (Table 2). Notably, the effect on cholesterol appeared to persist in the recovery period (Fig. 1). Glucose levels were not influenced by the treatments.

The only unique effect of the MXC treatments on clinical chemistry was an early effect on electrolyte balance. Sodium, chloride, and total bicarbonate (TCO<sub>2</sub>) concentrations were significantly higher in serum of MXC-treated monkeys than in the other two groups 3 months after initiation of treatment, (data not shown). This difference resolved by the next sampling time point 3 months later and did not reappear at later sampling times. However, sodium and TCO<sub>2</sub> were depressed in the MXC25 and DES groups at the end of treatment (Table 2).

### Lymphocytes

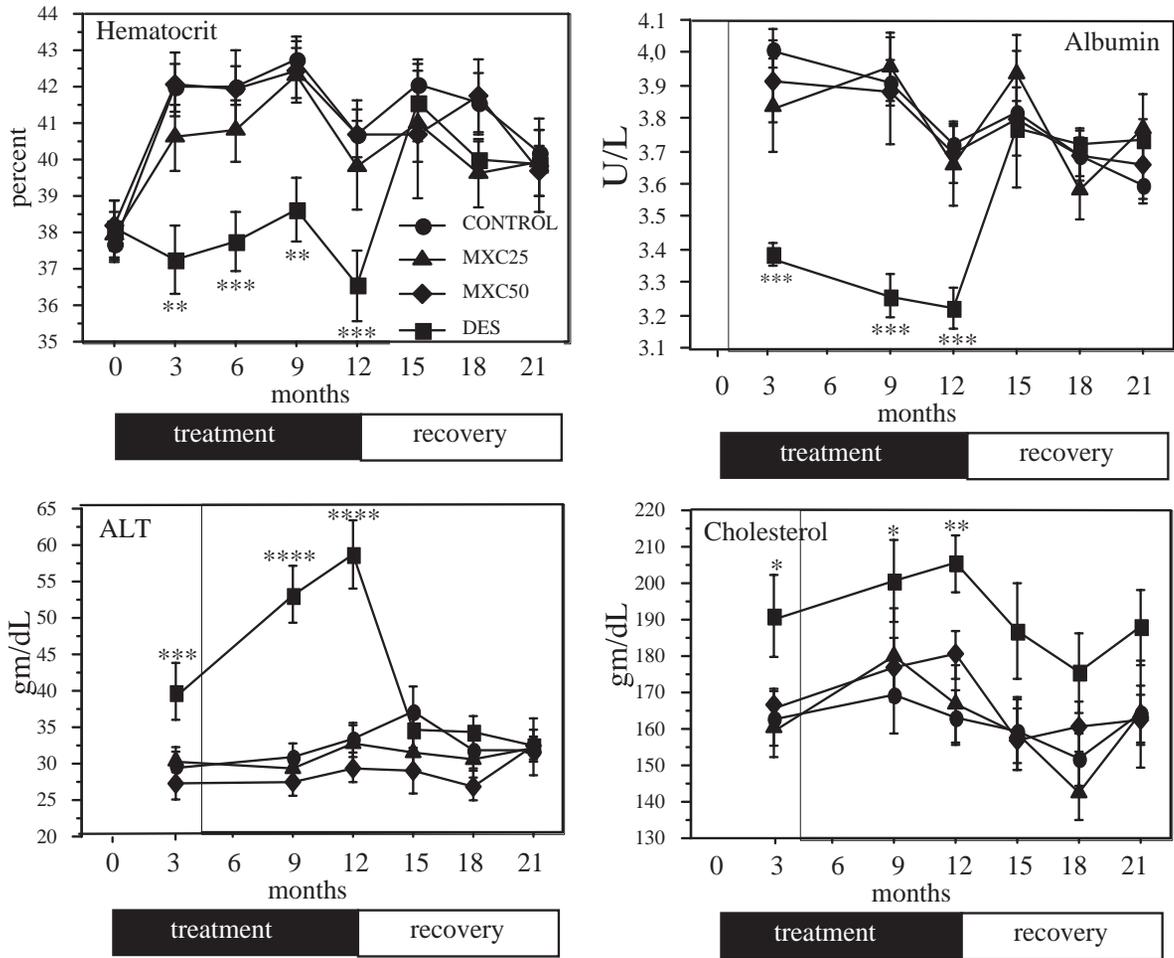
White blood cell (WBC) counts were slightly but not significantly depressed during the treatment period (data not shown). This depression was attributable to lymphocytes, which were reduced by about a third in the DES group relative to controls at the end of treatment (Table 1). After discontinuation of treatment, WBC counts remained lower than controls in the DES

group; this effect was statistically significant 3 and 6 months after discontinuation of treatment (data not shown). In particular, segmented neutrophil levels were elevated in all groups except the DES group at this time.

Further information on lymphocyte subpopulations was obtained from flow cytometry. The low population of B lymphocytes (CD20+/CD3-) was primarily responsible for the reduction in total lymphocytes (Table 1). The effects on B cells recovered by 3 months after discontinuation of treatment (Fig. 2). T-cells (CD3+) were also significantly reduced during treatment in the DES group (Fig. 2).

Absolute numbers of various T-lymphocyte populations were also generally reduced by the end of treatment in the DES group. The CD4+/CD29+ population was reduced in absolute numbers and as a percent of the lymphocytes in the MXC50 group (Table 3, Fig. 2). Another lymphocyte population, CD56+/CD3-, was also reduced in absolute numbers in the DES group at the end of the treatment period. This cell population showed a marked peak in numbers at 30 months of age, the average age at menarche in our colony (Fig. 2). The phenotype of this cell is not known. The CD56 protein is NCAM (neural cell adhesion molecule) and is a marker for circulating NK cells in humans, although this has not been supported in nonhuman primates (Carter *et al.*, 1999). We also assessed CD16+ as a potential NK marker (Giavedoni *et al.*, 2000; Munn *et al.*, 1996). Cells with CD3/8/16 markers, which have been proposed as NK markers in monkeys, had marginally, but not statistically significant, lower absolute numbers in the DES group when measured at the end of treatment and during recovery (Table 3).

An assessment of NK cell activity (chromium release assay) was performed at the end of the treatment period in the DES and



**FIG. 1.** Time course of puberty and treatment-related effects on hematocrit, albumin, ALT, and cholesterol.  $n = 8$  monkeys/group. Post-hoc  $t$ -test:  $*p \leq 0.05$ ,  $**p \leq 0.01$ ,  $***p \leq 0.001$ ,  $****p \leq 0.0001$ .

control groups only. Lysis of the specific NK target cell (K562) was somewhat lower in the DES than in the control group ( $p = 0.04$ ) at the highest effector-to-target cell ratio (100:1). However, lysis was also lower for the nonspecific target cell type (P815) in the DES group ( $p = 0.05$ ) at the 100:1 ratio. The effect may not have been specific to the NK targets, because the NK cells may have been activated to a phenotype with a broader range of cytotoxic efficacy (LAK cells).

Cytokine assays (IL-4, IL-10), intended to probe T-helper (TH)1/TH2 activity at the end of the treatment period, did not demonstrate differences between control and treated groups. Cytokine levels were highly variable. The only marginally significant effect was a greater ratio of IL-4 to IL-10 in the MXC25 group than in controls ( $p = 0.05$ ).

**Bone Mass**

Bone mass (bone mineral content, BMC, g; bone mineral density, BMD,  $g/cm^2$ ) was measured 9 months after discontinuation of treatment. At this time body weights were similar in all treatment groups, but long bone length were still

significantly lower in the DES group than in controls for all bones measured (femur, 6.7%; tibia, 4.7%; humerus, 5.8%; and radius, 1.4%) (Golub *et al.*, 2003).

The DEXA scans demonstrated that BMC was significantly lower in DES group than controls at the whole body level and in the lumbar spine, global proximal femur, and femur neck. BMD was lower in the DES group in all compartments measured, but group ANOVAS were statistically significant only for the femur (femoral neck and global proximal femur) (Fig. 3B).

Underlying changes in bone metabolism during treatment are suggested by the markedly depressed concentrations of calcium, phosphorous, and alkaline phosphatase from the clinical chemistry panel (Table 2, Fig. 3A) in the DES group. Phosphorous failed to rise in the DES group as it did in other groups around the time of puberty. Serum calcium did not show a puberty-related change in controls, but was depressed by the end of the third month of treatment in the DES group. After discontinuation of treatment, alkaline phosphatase rebounded to higher levels than controls, while calcium and phosphorous returned to control levels.

TABLE 2  
Mean Clinical Chemistry Values at the End of Treatment

	Group			
	Control	MXC 25 mg /kg/day	MXC 50 mg/kg/day	DES 0.5 mg/kg/day
Sodium (mM/l)	147 ± 0.4*	146 ± 0.9	147 ± 0.6	145 ± 1.2
Potassium (mM/l)	4.9 ± 0.2	5.3 ± 0.2	5.2 ± 0.3	5.1 ± 0.3
Chloride (mM/l)	103 ± 0.5	103 ± 0.6	103 ± 0.8	103 ± 1.0
Total CO <sub>2</sub> (mM/l)	24 ± 0.6	22 ± 0.8 <sup>a</sup>	23 ± 0.7	21 ± 0.7 <sup>b</sup>
Anion gap (mM/l)	26 ± 0.6	27 ± 0.9	27 ± 1.1	26 ± 0.9
Calcium (mg/dl)	10.6 ± 0.1	10.4 ± 0.1	10.4 ± 0.1	9.4 ± 0.1 <sup>d</sup>
Phosphorous (mg/dl)	5.2 ± 0.4	5.4 ± 0.4	5.9 ± 0.4	3.4 ± 0.3 <sup>b</sup>
Creatinine (mg/dl)	0.5 ± 0.01	0.5 ± 0.03	0.5 ± 0.04	0.5 ± 0.04
BUN (mg/dl)	30 ± 2	29 ± 1	27 ± 1	29 ± 2
Glucose (mg/dl)	80 ± 5	82 ± 3	83 ± 2	74 ± 4
ALT(SGPT) (U/l)	33 ± 2	32 ± 3	29 ± 2	58 ± 5 <sup>d</sup>
Alkaline Phosphatase (U/l)	475 ± 45	463 ± 50	504 ± 35	136 ± 16 <sup>d</sup>
Total Protein (g/dl)	7.3 ± 0.05	7.3 ± 0.2	7.3 ± 0.2	6.9 ± 0.1
Albumin (gm/dl)	3.7 ± 0.06	3.7 ± 0.1	3.7 ± 0.1	3.2 ± 0.07 <sup>c</sup>
GGT (U/l)	61 ± 4	62 ± 5	67 ± 5	61 ± 9
CPK (U/l)	456 ± 208	343 ± 185	404 ± 195	660 ± 295
AST(SGOT) (U/l)	26 ± 1	28 ± 1	25 ± 1	34 ± 2 <sup>c</sup>
Bilirubin (mg/dl)	0.2 ± 0.02	0.3 ± 0.04 <sup>a</sup>	0.2 ± 0.01	0.3 ± 0.04 <sup>a</sup>
LDH (U/l)	281 ± 24	287 ± 31	244 ± 27	304 ± 38
Cholesterol (mg/dl)	163 ± 8	167 ± 10	180 ± 7	205 ± 8 <sup>c</sup>
Triglycerides (mg/dl)	76 ± 6	130 ± 25 <sup>a</sup>	97 ± 9	125 ± 14 <sup>a</sup>

\*Mean ± SEM,  $n = 8$ /group.

<sup>a</sup>Significantly different from control, post-hoc  $t$ -test ( $p \leq 0.05$ ).

<sup>b</sup>Significantly different from control, post-hoc  $t$ -test ( $p \leq 0.01$ ).

<sup>c</sup>Significantly different from control, post-hoc  $t$ -test ( $p \leq 0.001$ ).

<sup>d</sup>Significantly different from control, post-hoc  $t$ -test ( $p < 0.0001$ ).

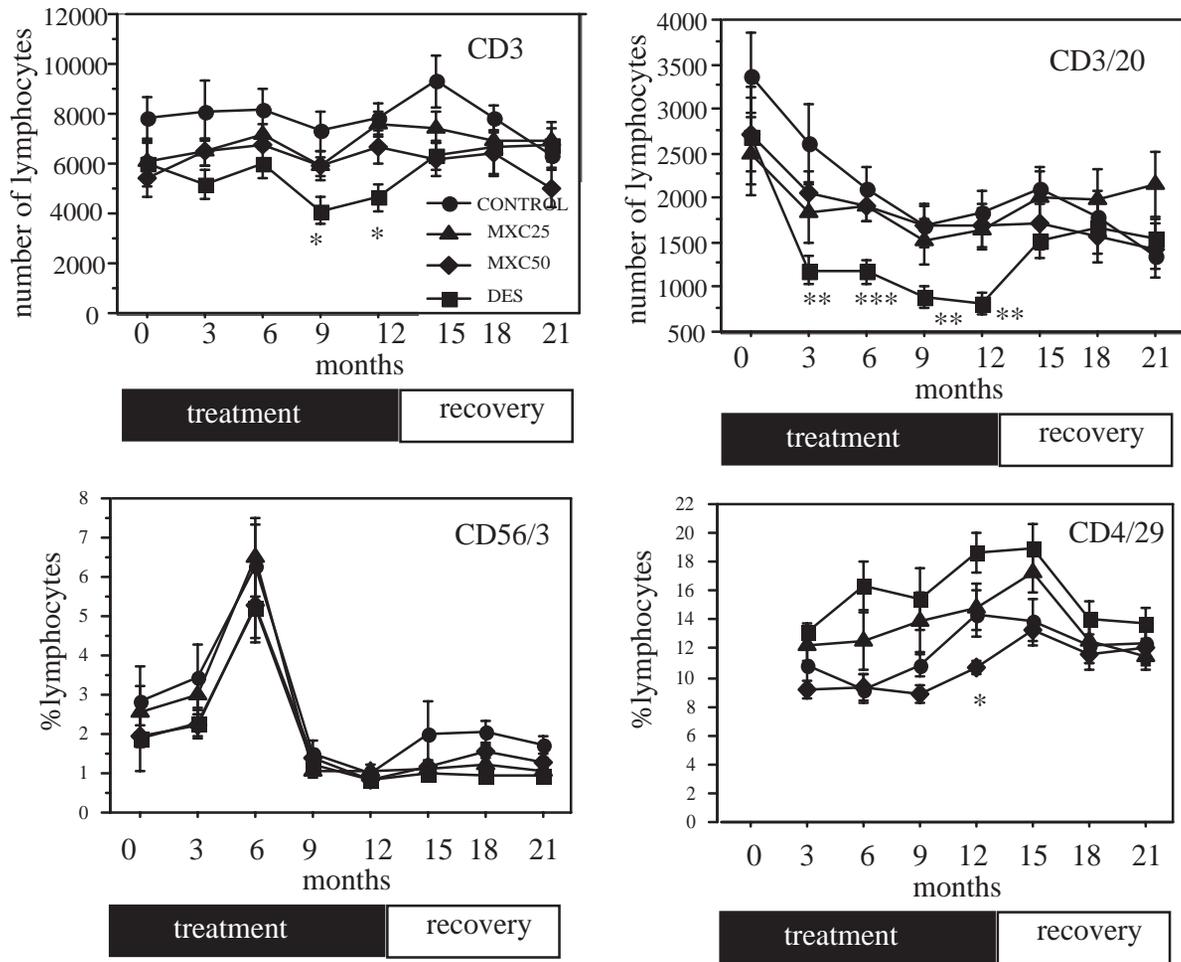
Interestingly, in the MXC50 group, BMD in the femur neck and global proximal femur was also significantly lower than in controls (Fig. 3B), although calcium, phosphorous, and alkaline phosphatase did not show any alteration during treatment (Fig. 3A).

## DISCUSSION

Across data sets, the DES group was most markedly and consistently affected by treatment. This agrees with our previous findings in this cohort of the greater estrogenicity of the DES treatment than the MXC treatments, as demonstrated in a receptor activation assay, as well as in growth and reproductive tract maturation (Golub *et al.*, 2003). Similar but smaller effects were sometimes seen in the MXC25 and MXC50 groups, not always in a dose-dependent pattern. As reported previously, the MXC25 group was sometimes more affected than the MXC50 group. This may be related to the different estrogen receptor affinities of the two agents. As demonstrated in *in vitro* receptor activation studies, DES is an agonist at both ER $\alpha$  and ER $\beta$  receptors. MXC, via its estrogenic metabolite HPTE, can have an ER $\beta$  antagonist effect (Gaido *et al.*, 1999, 2000; Waters *et al.*, 2001).

Most of the changes in circulating blood cells represent adjustments in the normal range and did not result in clinically relevant profiles. Neither anemia nor immunosuppression was indicated. Some of these effects were superimposed on maturational trends and could potentially be interpreted as altered maturation or altered sexual differentiation. Because extensive normative data on pubertal male and female rhesus is not available, it is difficult to adopt this interpretation.

Some, but not all, effects on clinical chemistry values can be characterized as estrogenic, based on other studies and clinical reports (Christiansen and Riis, 1990; Cowan *et al.*, 1982; Gray *et al.*, 1989; Kivinen and Maenpaa, 1990; Schiele *et al.*, 1998; Walden *et al.*, 1986). Various studies of oral contraceptives and hormone replacement therapy consistently report depression of alkaline phosphatase and bilirubin values from clinical chemistry panels. Similarly, elevated cholesterol and triglycerides are common in these studies. The depression in albumin in the DES group in this study was quite severe and could profitably be explored in terms of mechanism and clinical consequences. Although less frequently reported, there are some data suggesting that contraceptives can lower plasma albumin. Contraceptives are also associated with microalbuminuria, a moderate



**FIG. 2.** Time course of puberty and treatment-related effects on lymphocyte populations obtained by flow cytometric analysis. *n* = 8 monkeys/group. Post-hoc *t*-test: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001.

increase in albumin excretion measured as a marker of vascular damage and cardiovascular risk (Monster *et al.*, 2001). The impact on circulating albumin of microalbuminuria was not discussed.

Estrogen therapy effects on serum liver transaminases are less consistent; some papers identified reductions (Cowan *et al.*, 1982; Kivinen and Maenpaa, 1990), while others identified increases (Walden *et al.*, 1986) that were attributed to liver toxicity. Dose may be an important consideration, but no dose-response data are available in the human literature. While transaminases are traditionally measured as an index of liver toxicity, it is possible that estrogenic effects represent a metabolic regulatory effect. The rapid reversal of the effects in our study after discontinuation of treatment suggests a transient effect, possibly related to estrogen regulation rather than liver damage.

Other effects can also be considered indicative of direct estrogen activity. Estrogen is known to inhibit hematopoiesis and enhance bone turnover through effects on bone marrow (Erben *et al.*, 1998; Jilka *et al.*, 1995; Manolagas *et al.*,

1995). Estrogen effects on B-cell generation have been widely studied (Erben *et al.*, 1998; Erlandsson *et al.*, 2002, 2003; Kincade *et al.*, 2000; Perry *et al.*, 2000).

Contraceptives are known to enhance bone mass in adult women, and estrogen improves bone density of postmenopausal women. However, bone mass is adversely affected by contraceptive treatment in younger women who have not yet reached peak bone mass (Polatti *et al.*, 1995), and also in young postpubertal monkeys (Register *et al.*, 1997). Serum calcium and alkaline phosphatase were lower than normal in young contraceptive-treated monkeys (Register *et al.*, 1997), as was the case in the DES group in the present study. In our study, the reduction in bone mass after 12 months of DES treatment (and 6 months recovery) was 3–5 times greater than in young monkeys after 10 months of oral contraceptive treatment (Register *et al.*, 1997). These effects are in part attributable to inhibition of growth in bone length, resulting in smaller bones of lower total mineral mass, but the reduction in BMD as well as BMC in the proximal femur suggests a possible additional effect on the structure or mineralization of the bone. MXC50

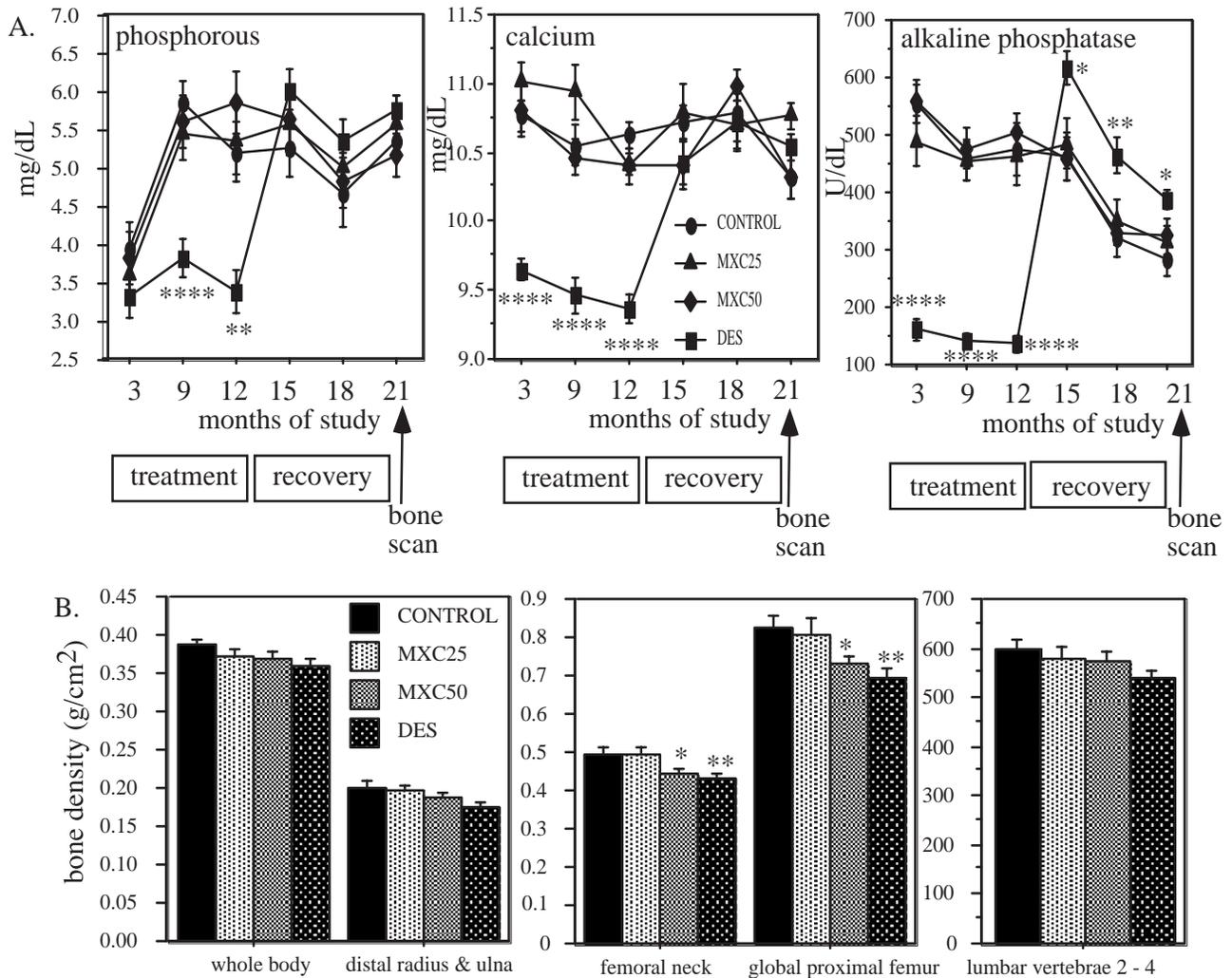
**TABLE 3**  
**Absolute Numbers of Peripheral Lymphocytes by Flow Cytometry at the End of Treatment**

	Group			
	Control	MXC 25 mg/kg/day	MXC 50 mg/kg/day	DES 0.5 mg/kg/day
CD4+/CD3+	2908 ± 272*	2936 ± 310	2336 ± 249	2013 ± 296 <sup>a</sup>
CD8+/CD3+	1669 ± 164	1843 ± 174	1401 ± 267	964 ± 104 <sup>b</sup>
CD4, CD8 ratio	1.8 ± 0.1	1.6 ± 0.2	1.9 ± 0.2	2.1 ± 0.2
CD56+/CD3-	78 ± 13	78 ± 14	55 ± 10	37 ± 6 <sup>a</sup>
CD20+/CD3-	1838 ± 252	1648 ± 234	1685 ± 250	815 ± 129 <sup>b</sup>
CD4+/CD45+	2055 ± 211	2060 ± 240	1596 ± 235	1303 ± 182 <sup>a</sup>
CD4+/CD29+	1131 ± 154	1131 ± 147	703 ± 61 <sup>a</sup>	837 ± 85
CD3-/8+/16+	619 ± 38	746 ± 247	420 ± 93	349 ± 72

\*Mean ± SEM.

<sup>a</sup>Significantly different from control, post-hoc *t*-test ( $p \leq 0.05$ ).

<sup>b</sup>Significantly different from control, post-hoc *t*-test ( $p \leq 0.01$ ).



**FIG. 3.** Effects of exogenous estrogens on bone density. (A) Time course of changes in phosphorous, calcium, and alkaline phosphatase during and after treatment. Values from clinical chemistries obtained at 3 month intervals beginning 3 months after initiation of treatment. (B) Bone mineral density from DEXA scan taken 9 months after discontinuation of treatment (month 21 of study).  $n = 8$  monkeys/group. Post-hoc *t*-test: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ .

reduced bone mass without effect on bone length, suggestive of an effect of this agent on structure or mineralization of bone, independent of a growth effect.

Two lymphocyte subpopulations demonstrated interesting, apparent puberty-related changes in this population of young female rhesus. Cells displaying the CD56 marker demonstrated a distinct peak in numbers at 30 months of age, the anticipated average age at menarche, when they represented 6% of the lymphocyte population. Subsequently, this number declined to about 1% and remained stable at this level for the rest of the study. The absolute numbers of this cell type were reduced by about half in the DES group at the end of treatment. CD56 (NCAM) is a marker for circulating NK cells in humans, but not in monkeys (Carter *et al.*, 1999). However, CD56 is a marker for endometrial NK cells in both rhesus and humans (Slukvin *et al.*, 2001). In this experiment, CD56 may be a marker for a unique lymphocyte population that is important for immune function/maturation at the time of puberty. For example, this population may be involved in seeding the reproductive tract in connection with establishment of mucosal immunity. However, no information is currently available on the characteristics and functions of this lymphocyte subpopulation.

A second lymphocyte population that showed distinct puberty-related changes, as well as an influence of exogenous estrogen, displayed CD4 and CD29 markers. CD4/CD29 cell numbers, as a percent of lymphocytes, began to rise around 30 months of age, reaching 50% higher levels in the next 6 months. This rise was influenced by the exogenous estrogen treatment; the percent of cells increased earlier and reached a higher level in the DES and MXC25 groups, but was slower to rise in the MXC50 group. After discontinuation of treatments, all groups had similar numbers of CD4/CD29 lymphocytes. The possibility that sex-differentiation in this lymphocyte population is established at puberty is supported by data (Rudy *et al.*, 2002) demonstrating higher CD4/CD29 counts in female than in male adolescents in a longitudinal study conducted from 15 to 20 years of age. Long-term age-related changes in this phenotype, which are associated with resting memory T-helper cells, have been reported in cynomolgous monkeys (Lee *et al.*, 2003), as well as in humans and mice (Thoman, 1995; Utsuyama *et al.*, 2002). Increased numbers of CD4/CD29 cells are associated with an increase in circulating CD4/CD8 double positive (DP) cells and T-cell receptor excision circles (TRECS), which are thought to reflect thymic involution (Sodora *et al.*, 2000). Interestingly, there were indications that the DP cells arise from a phenotypic alteration of the CD4/CD29 SP cells. However, it is not clear what a short-term reversible change in this cell population, such as that seen in the present experiment, would represent in terms of immune system maturation and function.

Effects of exogenous estrogens as detected in this study could be interpreted as toxicity, as estrogen regulation, or as altered sexual maturation. Further data would be needed to distinguish these possibilities. Parameters that do not show a

distinctive developmental pattern and reverse immediately upon discontinuation of treatment, such as effects on liver transaminases and albumin, may reflect reversible toxicity. Parameters that show distinct puberty-related changes in controls that are altered in treated monkeys, like bone mineralization and T-helper memory cell population increases, may reflect disrupted development. In general, any parameter changes with excursions outside the normal range as represented by the control group can be considered as adverse with implications for health.

Absent from our study is characterization of estrogen effects in this study population across a range of doses. Practical limitations in conducting studies with large group sizes in nonhuman primates limited our dose selection to the higher dose range.

Because of extensive feedback regulation and receptor cross reactivity, dose-response relationships for endocrine disruptors are difficult to predict without empirical data. In particular low-dose effects may differ both qualitatively and quantitatively from those in a moderate range, such as is encountered with endocrine therapies, or the high doses that are explored in typical toxicology studies. However, it should be noted that all animals remained healthy during the study and no mortality or morbidity was encountered. Food intake was not affected.

Of potential public health significance is the different profile of the two agents, DES and MXC. While DES was much more effective in disrupting pubertal growth and menarche (Golub *et al.*, 2003), MXC was more effective in disrupting behavior (Golub *et al.*, 2004). In the current report, DES had greater effects on immunohematology, but DES and MXC similarly impacted bone mass. This suggests that health risk of exogenous estrogen exposure in adolescence is not readily generalizable across agents.

Regardless of the characterization of the effects, possible links to later health risks are of great interest. Although many of these estrogenic effects have already been documented in connection with estrogen therapies, corresponding health risks in human populations are not necessarily appreciated. For example, effects of estrogen on fibrinogen have been known for a number of decades, but consequences for fatal clotting incidents were not recognized until full-scale randomized trials of hormone replacement therapy were undertaken. In considering the toxicology of environmental estrogens, which have no therapeutic role, identification of long-term health risks are even more important, and nonhuman primate models can play an important role.

As regards endocrine disruption in adolescence, possible examples of long-term health risk are osteoporosis in the case of bone mineralization effects, autoimmunity in the case of shifts in lymphocyte populations, and cardiovascular disease in the case of cholesterol and triglyceride elevation. Red blood cell effects appeared to recover completely; however hematological effects could be considered a risk factor for anemia, a common problem in adolescent girls.

In summary, attention to the effects of endocrine disruption has focused on the reproductive system. However, long-term effects in a variety of systems relevant to future health could

be altered when exogenous estrogens impinge on sensitive developmental periods. Some potential health impacts of estrogen disruption in adolescence include susceptibility to autoimmunity, heart disease, anemia, and osteoporosis.

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