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IMMUNITY IN CHILDREN WITH EXPOSURE TO ENVIRONMENTAL LEAD: I. EFFECTS ON CELL NUMBERS AND CELL-MEDIATED IMMUNITY.

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ABSTRACT

Studies conducted in animal systems have shown that lead is an immunosuppressive agent at levels far below those causing overt toxicity. Children less than six years of age are the population at highest risk for exposure to environmental lead; however little data were available to assess effects on the developing immune system in this age group. Reported here is the completed Phase I study on 193 children, ages 9 months to 6 years, with blood lead levels from 1 to 50 μg/dl, recruited from the urban population of Springfield-Greene County, Missouri, through their participation in the WIC (Women, Infants, and Children) and Lead Poisoning Prevention Programs. This portion of the study dealt with enumeration of cells involved with the immune response and in vitro mitogenic stimulation of lymphocytes. The percent lymphocytes, monocytes, granulocytes, T cells (total), B cells, T_H's and T_S's and T_H/T_S ratios were determined and the data were analyzed. No consistent significant differences were seen among the various risk categories, which conform to current CDC guidelines. Though two age groups showed some possible effect of lead, none of the various cellular parameters within these age groups showed significant correlation with blood lead. The lymphocyte response to in vitro mitogenic stimulus was studied on 42 children (including 17 in risk classifications IIA and higher) using the mitogens phytohemagglutinin (PHA), Concanavalin A (Con A), and Pokeweed mitogen (PWM). No consistent statistically significant differences were seen among the various risk categories; the effects of lead, if present, are most likely subtle and obscured by the interindividual and time-dependent variation inherent in this type of study.

INTRODUCTION

Concern over the human health impact of lead exposure has in recent years broadened to include not only acute toxicity (> 50 μg/dl blood lead) but also the insidious chronic health effects from moderate or even low level exposure (10-25 μg/dl BPb). In young children, neurobehavioral and cognitive impairment has been documented at dentin lead levels as low as 6.0-8.2 ppm (Needleman *et al.*, 1990). In light of the growing body of evidence, the Agency for Toxic Substances and Disease Registry lowered the threshold for neurobehavioral toxicity to 10-15 μg/dl BPb in 1988 (ATSDR, 1988). In October of 1991, the Center for Disease Control adjusted the risk classifications for childhood lead exposure and have lowered their suggested intervention level to 10-15 μg/dl BPb as well (CDC, 1991) (Table 1).

The effects of lead on human immunity are not well documented. Studies in animal systems have shown impaired host resistance to bacterial, viral, and parasitic infections after exposure to lead (Hemphill *et al.*, 1971; Gainer, 1977; Agrawal *et al.*, 1989). Susceptibility to endotoxin and oncogenic challenge is also enhanced (Dentener *et al.*, 1989; Kerkvliet and Baecher-Steppan, 1982). The available data on the effects of lead on human host resistance suggest that resistance is lowered (Ewers *et al.*, 1982; Sachs, 1978). Both the humoral (B cell-mediated) and cellular (T cell-mediated) "arms" of the immune system have been studied in an attempt to elucidate the exact cell type(s) affected. Data concerning cellular responses are briefly reviewed here; humoral responses are discussed in the accompanying poster (Lutz *et al.*, 1992b).

A reduction in the *in vivo* delayed-type hypersensitivity response of T cells in rodents (measured by lymphokine production) was found after chronic lead exposure at even low levels (Muller *et al.*, 1977; Faith *et al.*, 1979). However, the choice of lead salt was critical (Descotes *et al.*, 1984; Laschi-Loquerie *et al.*, 1984). *In vitro* DTH responses in mice were analyzed by measuring proliferation in a mixed lymphocyte culture (MLC). Lead was found to decrease the response in one case (Koller and Roan, 1980) and to have no effect in the other (Lawrence, 1981). The proliferative response to mitogens (lectins or carbohydrate-binding proteins) is another measure of cellular immunity. *In vitro* mitogen stimulation assays following either *in vivo* or *in vitro* exposure to lead have been reported. In such animal studies lead has been found to inhibit (Dentener *et al.*, 1989; Ewers *et al.*, 1982), to stimulate (Sachs, 1978), and to have no effect (Gainer, 1973; Gainer, 1974). [These data are difficult to correlate due to differences in concentration of lead, differences in the lead salt used, *in vivo* vs *in vitro* exposure to lead, and technical aspects of the stimulation assays (mitogen used, for example).] Data on cellular immunity in humans following lead exposure are sparse.

As public policy decisions are made on permissible levels of lead in the human environment, and in particular the environment of children, more information is needed on the effects of lead on other organ systems. In this study and the companion study of humoral immunity, a comprehensive survey of the developing immune system in children (ages 9 months to 6 years) exposed to environmental lead has begun. The population, along with controls, was chosen from participants in the WIC (Women, Infants, and Children) Program of nutritional support in the urban environment of Springfield-Greene County, Missouri (population 140,000). Lead contamination in this environment most often is due to lead-based paint remaining on the interior and exterior walls of the child's residence. Reported in this portion of the Phase I study are both attempts to ascertain the effects of lead on absolute numbers of the various cell types involved in an immune response and assays of cell function in the form of the lymphocyte response to mitogens. [Phytohemagglutinin (PHA) and Concanavalin A (Con A) which stimulate human T cells and Pokeweed mitogen (PWM) which stimulates both B and T cells in humans were used.] Such a systematic survey of immune function in a population where blood lead levels span all risk categories will aid in determining the health risk of moderate to low level lead exposure.

Table 1. Risk Classification of Children Based on [blood Pb].*

Class	Blood Pb (μg/dl)	Comment
I	< 9	Not considered to be lead-poisoned.
IIA	10-14	If many children in this range, community-wide childhood lead poisoning prevention activities should be triggered. Children in this range need frequent rescreening.
IIB	15-19	Nutritional and educational interventions and more frequent screening are necessary. If blood lead level persists in this range, an environmental investigation and intervention should be done.
III	20-44	Environmental evaluation and remediation, and a medical evaluation are needed. Pharmacologic treatment of lead poisoning may be necessary.
IV	45-69	Medical and environmental interventions are needed, including chelation therapy.
V	>70	This is considered to be a medical emergency. Medical and environmental management should begin immediately.

* Taken from the new CDC statement on preventing lead poisoning in young children (October 8, 1991).

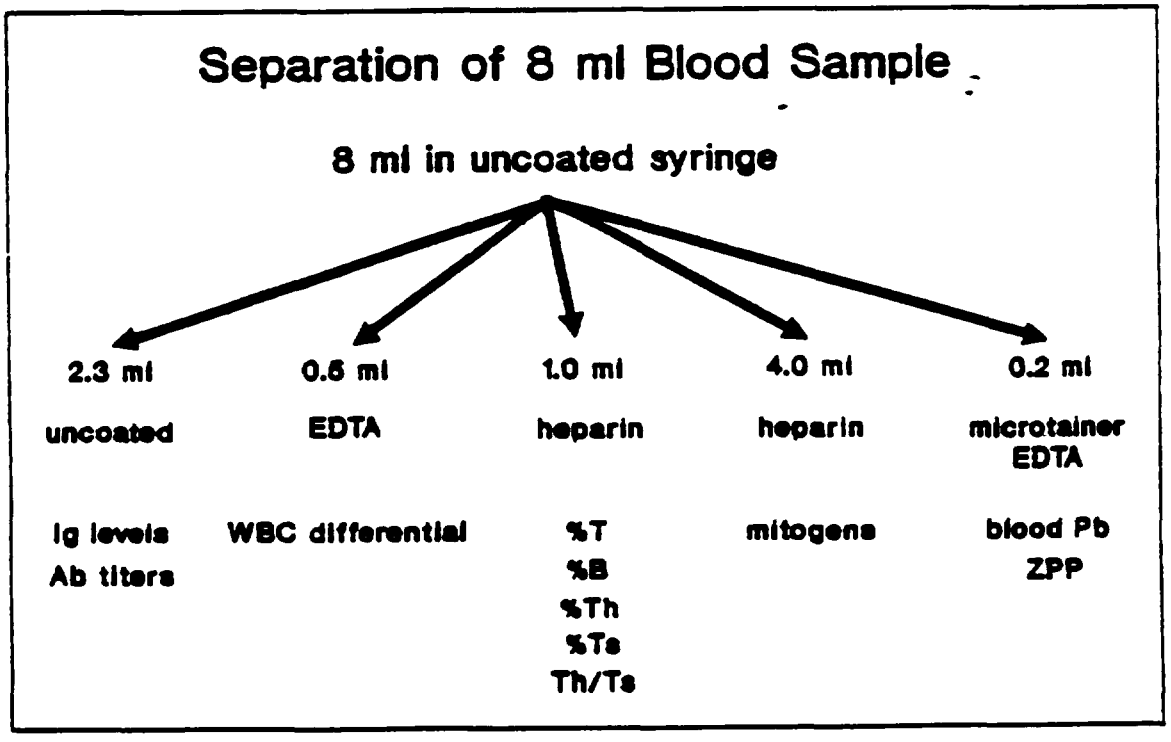

Figure 1

MATERIALS/METHODS

Handling of blood sample

The study poplulation consisted of children, 9 months to 6 years of age, who were recruited through their participation in the Women, Infants, and Children (WIC) program of nutritional support and the Childhood Lead Poisoning Prevention Program in Springfield-Greene County, Missouri. Studies were performed on venous blood samples, drawn by a trained pediatric phlebotomist at the WIC office or during home visits. The blood sample was taken with a 22-gauge needle and an uncoated 10cc syringe, and quickly separated into aliquots for various assays (Figure 1). Coated, lead-free vacutainers (B-D) were used for the aliquots. Samples were held at room temperature and transported to St. John's Regional Medical Center (Springfield) or the University of Missouri-Rolla by car.

WBC differential counts

A white blood cell differential was performed at St. John's Regional Medical Center on blood held in EDTA. Complete blood count (CBC) data was determined by a Coulter Stack S (fully automated), and the percent lymphocytes, monocytes, and granulocytes selected from that data. Counts are made on the basis of size and granularity.

Cytofluorimetry

The percent T, B, T_H, and T_S, and the T_H/T_S ratio were determined by cytofluorimetry analysis at St. John's CAP-certified flow cytometry laboratory, which has a Coulter Epics Profile I with a 15 mW argon laser. Blood held in heparin was used for these assays. Aliquots were treated with a panel of Coulter Cyto-Stat monoclonal antibodies directed against markers found on the surface of these lymphocyte classes and subclasses. T and B cells were distinguished using T11-RD1/B4-FITC [T11 = CD2, the sheep erythrocyte receptor found on T cells (Reinherz and Schlossman, 1982); B4 = CD 19, found on normal B cells (Nadler *et al.*, 1983); RD1 = phycoerythrin (red); FITC = fluorescein (green)]. T_H and $T_{S/C}$ were distinguished using T4-RD1/T8-FITC [T4 = CD4 (Reinherz *et al.*, 1979); T8 = CD8 (Reinherz *et al.*, 1980)]. MsIgG1-RD1 or MsIgG1-FITC was used as the non-specific mAb control. Mo2-RD1/KC56 (T200)-FITC was the control for the accuracy of the 90° light scatter and forward angle light scatter used to set the gates for flow cytometric analysis [Mo2 = CD14, a human myeloid antigen (Todd *et al.*, 1981); KC56 = CD45, a pan leukocyte antigen (Newman *et al.*, 1984)]. After the addition of the antibodies and incubation, red cells were lysed and the remaining cells washed via the Coulter Quik-Prep procedure. The samples were then run on the flow cytometer and five thousand events were measured per assay.

Mitogen stimulation assays

Mononuclear cells were isolated from 4 ml of heparinized blood on Ficoll gradients (LSM, Lymphocyte Separation Medium; Organon Teknika) and suspended in RPMI 1640 at the cell concentration of 1×10^6 cells/ml. Samples were dispensed into round bottomed microtitre plates at cell concentrations of 2.5×10^5 and 1×10^5 cells/well. [These cell concentrations do not give maximal measured blast cell transformation; however, the literature indicates subtle changes in responsiveness could be more easily detected at limiting cell concentrations. There was also concern about the number of lymphocytes which could be obtained from 4 ml of blood.]

Appropriate concentrations of each mitogen and days of peak ^{3}H-thymidine uptake were determined using control lymphocytes (data not shown). For PHA (a T cell mitogen) in this laboratory, final concentrations of 10μl and 5μl/ml of a suspension obtained from Difco give peak proliferation on days 3 and 4. For ConA (a T cell mitogen which shows preference for CD8+ cells)(Sigma), 40 and 20μg/ml give peak responses also on days 3 and 4. For PWM (Gibco), 2 and 1μl/ml give peak responses on days 5 and 6. [NOTE: The PHA and PWM preparations are not purified; their concentrations are expressed in terms of an activity when purchased. Concentrations shown are dilutions of these preps.]

Six hours prior to harvest 1 μCi of ^{3}H-thymidine was added to each well. The plates were harvested onto filtermats using the Skatron semiautomatic cell harvester. Data is displayed in the form of mean ± standard deviation of the counts per minute of quadruplicate samples.

RESULTS/DISCUSSION/CONCLUSIONS

Tables 2-6 show the statistical analysis of the data on the per cent lymphocytes, monocytes, granulocytes, T cells (total), B cells, T_H's, and T_S's, and the T_H/T_S ratios, grouped according to age (9-15 months, 16-22 months, 23-29 months, 30-36 months, and 37 + months). Shown in each table for each parameter are p values for the chi squared analysis to detect statistically significant differences among the risk categories in that age group. Directly beneath the chi squared p values are the correlation coefficients for the correlation of each parameter with raw blood lead values, and the p value for that correlation. [Correlations were performed according to the method of Spearman for a non-normal distribution (Snedecor and Cochran, 1980)]. Risk categories used are those recommended by the CDC in 1991 (CDC, 1991) (Table 1).

For virtually all of these assays in all age groups, no statistically significant differences were noted [p value (confidence limit for differences among risk groups) > 0.05]. These data suggest that lead will have no effect on these parameters; however, caution is necessary in interpretation since sample sizes in the higher risk groups were not large. (In some age groups, the population in a particular risk group was small to nonexistent.)

In the 16 to 22 month age group (Table 3), two parameters showed or approached significant differences among the risk categories: % T_S's ($p = 0.06$) and T_H/T_S ratios ($p = 0.05$). The parameters behaved erratically however, and showed no correlation with raw blood Pb (BPb) values within this age group [p values for the correlations with BPb are 0.54 and 0.25, respectively]. The 23-29 month age group (Table 4) showed a suggestion of a difference among the risk groups in % T cells (total) ($p = 0.07$), % lymphocytes ($p = 0.13$), and % granulocytes ($p = 0.13$). Again, the confidence limits for the correlations of these parameters with BPb did not suggest a strong correlation ($p = 0.17$ for the correlation of BPb and % T's; $p = 0.14$ for BPb and lymphocytes; $p = 0.18$ for BPb and granulocytes). These data agree with preliminary conclusions reported on the first 118 children in the study (Lutz *et al.*, 1992b). Despite the restructuring of risk categories to reflect the new CDC guidelines and the increase in the population size, no strong correlations were seen between BPb and any parameter in any age group.

Figures 2-8 show data generated from *in vitro* mitogen stimulation assays performed on a subset of children in this study. In all, lymphocytes from 42 children were stimulated with the mitogens phytohemagglutinin (PHA), Concanavalin A (Con A), and Pokeweed mitogen (PWM). (Seventeen of these children were ranked in risk class IIA and higher.) Three mitogens were used to obtain a more complete picture of lymphocyte function. PHA and Con A stimulate human T cells (Andersson *et al.*, 1972) while PWM is mitogenic for both B and T cells in humans (Boerjeson *et al.*, 1966). Results shown represent the mean of quadruplicate samples, with standard deviations indicated for each bar. The "RPMI" data are the media control in each assay (no mitogen added). Statistical analysis was performed on each assay and correlation coefficients (Spearman) for blood lead and uptake of ^{3}H-thymidine were generated for each concentration of each mitogen on each day of harvest. (Inherent variation in normal control data precluded the comparison of individuals between assays performed on different days--see below.)

In Figure 2, data from the first three children and one adult control are shown. In this assay, for PHA, an increase in blood lead value did appear to correlate with a decreased lymphocyte response ($p = 0.05$); patient A with a BPb of 20 μg/dl (Class III) showed the lowest response, measured in cpm of ^{3}H-thymidine taken up by the proliferating cells. However, no other statistically significant correlations were seen between BPb and uptake of labelled thymidine in the remaining 10 assays performed. There was no discernable difference in the response of children with elevated blood lead. Figures 3-6 show PHA, Con A, and PWM stimulations from selected assays. In Figure 3, with three Class IIB children and one Class III, there was no correlation between BPb and decreased stimulation ($p > 0.05$). Likewise in Figure 4 (MNOPQ Day 3), Patient P with a BPb of 50 μg/dl (Class IV) showed no decrease in stimulation when compared to the Class I children or the adult control in that assay. In Figure 5 (14-17 Day 3), four higher risk class children (BPb's of 14 and 15 μg/dl, Class IIA; BPb of 15, Class IIB; BPb of 29, Class III) are compared with an adult control. Uptake of labelled thymidine did not correlate with BPb ($p > 0.05$). Figures 6 and 7 show PWM data for two assays,

Table 2: Cellular Data for Ages 9 to 15 Months

Risk Cat.	N	%Lymph Mean S.D.	%Mono Mean S.D.	%Gran Mean S.D.	$\%T_H$ Mean S.D.
I	28	58.1 ± 15.0	7.6 ± 2.4	34.3 ± 14.0	47.2 ± 7.8
IIA	8	63.1 ± 4.4	6.4 ± 1.0	30.6 ± 4.1	48.6 ± 9.2
IIB	4	51.6 ± 8.0	8.0 ± 2.1 (3)	37.1 ± 7.9 (3)	45.9 ± 4.4
III	10	63.1 ± 11.6 (9)	8.0 ± 1.6 (9)	28.7 ± 10.4	48.3 ± 7.1
		p[a] = 0.26	p = 0.29	p = 0.48	p = 0.71
Corr. Coeff.[b]		r = -0.01	r = 0.12	r = -0.05	r = 0.06
		p[c] = 0.94	p = 0.41	p = 0.73	p = 0.69

Risk Cat.	N	$\%T_S$ Mean S.D.	T_H/T_S Mean S.D.	%T(total) Mean S.D.	%B cells Mean S.D.
I	28	17.5 ± 5.1	3.1 ± 1.5	71.8 ± 6.4	23.3 ± 7.6
IIA	8	18.3 ± 4.6	2.7 ± 0.6	72.3 ± 7.1	26.5 ± 7.7
IIB	4	15.3 ± 3.5	3.2 ± 0.9	72.3 ± 5.9	26.5 ± 5.6
III	10	15.2 ± 2.7	3.3 ± 0.7	70.6 ± 5.0	25.4 ± 4.9
		p[a] = 0.24	p = 0.54	p = 0.94	p = 0.56
Corr. Coeff.[b]		r = -0.09	r = 0.06	r = 0.02	r = 0.15
		p[c] = 0.56	p = 0.69	p = 0.88	p = 0.29

[a] Confidence limits for differences among risk groups.
[b] Spearman correlation coefficient for blood lead (BPb).
[c] Confidence limits for correlation with BPb.

Table 3: Cellular Data for Ages 16 to 22 Months

Ri Cat.	N	%Lymph Mean S.D.	%Mono Mean S.D.	%Gran Mean S.D.	$\%T_H$ Mean S.D.
I	25	57.2 ± 9.6	7.4 ± 2.2	35.4 ± 9.2	46.0 ± 6.9
IIA	6	63.2 ± 5.3	7.0 ± 2.1	29.9 ± 6.0	45.4 ± 5.1
IIB	5	59.6 ± 12.0	7.8 ± 1.9	32.6 ± 12.3	48.5 ± 7.1
III	4	49.8 ± 16.2	9.4 ± 2.2	40.8 ± 14.7	42.1 ± 5.7
		p[a] = 0.34	p = 0.33	p = 0.53	p = 0.44
Corr. Coeff.[b]		r = -0.07	r = 0.06	r = 0.06	r = -0.16
		p[c] = 0.69	p = 0.71	p = 0.71	p = 0.32

Risk Cat.	N	$\%T_S$ Mean S.D.	T_H/T_S Mean S.D.	%T(total) Mean S.D.	%B cells Mean S.D.
I	25	17.9 ± 3.8	2.7 ± 0.8	71.4 ± 5.6	26.5 ± 5.4
IIA	6	17.6 ± 6.9	3.0 ± 1.3	72.0 ± 5.4	27.2 ± 6.9
IIB	5	13.5 ± 2.9	3.8 ± 1.2	70.6 ± 4.2	26.4 ± 4.1
III	4	21.4 ± 3.3	2.3 ± 0.5	69.4 ± 6.0	25.9 ± 4.2
		p[a] = 0.06	p = 0.05	p = 0.78	p = 0.90
Corr. Coeff.[b]		r = 0.10	r = -0.19	r = -0.08	r = -0.02
		p[c] = 0.54	p = 0.25	p = 0.63	p = 0.89

[a] Confidence limits for differences among risk groups.
[b] Spearman correlation coefficient for blood lead (BPb).
[c] Confidence limits for correlation with BPb.

Table 4: Cellular Data for Ages 23 to 29 Months

Risk Cat.	N	%Lymph Mean S.D.	%Mono Mean S.D.	%Gran Mean S.D.	$\%T_H$ Mean S.D.
I	21	54.8 ± 9.4	7.9 ± 1.9	37.3 ± 8.8	46.8 ± 9.0
IIA	10	51.4 ± 11.8	8.2 ± 1.7	40.3 ± 11.0	41.7 ± 6.5
III	2	34.0 ± 14.4	6.9 ± 0.4	59.2 ± 13.9	40.7 ± 3.3
		p[a] = 0.13	p = 0.64	p = 0.13	p = 0.19
Corr. Coeff.[b]		r = -0.26	r = 0.05	r = 0.24	r = -0.25
		p[c] = 0.14	p = 0.76	p = 0.18	p = 0.16

Risk Cat.	N	$\%T_S$ Mean S.D.	T_H/T_S Mean S.D.	%T(total) Mean S.D.	%B cells Mean S.D.
I	21	17.1 ± 5.2	3.0 ± 1.1	71.9 ± 6.5	25.0 ± 6.8
IIA	10	18.2 ± 4.6	2.5 ± 1.1	67.8 ± 3.8	29.7 ± 6.8
III	2	22.6 ± 0.6	1.8 ± 0.1	73.2 ± 5.4	22.3 ± 8.1
		p[a] = 0.17	p = 0.18	p = 0.07	p = 0.23
Corr. Coeff.[b]		r = 0.24	r = -0.26	r = -0.24	r = 0.11
		p[c] = 0.18	p = 0.15	p = 0.17	p = 0.56

[a] Confidence limits for differences among risk groups.
[b] Spearman correlation coefficient for blood lead (BPb).
[c] Confidence limits for correlation with BPb.

Table 5: Cellular Data for Ages 30 to 36 Months

Risk Cat.	N	%Lymph Mean S.D.	%Mono Mean S.D.	%Gran Mean S.D.	$\%T_H$ Mean S.D.
I	6	42.4 ± 6.8	7.6 ± 1.6	50.1 ± 6.1	41.8 ± 7.9
IIA	3	36.2 ± 18.1	8.7 ± 1.5	55.1 ± 16.6	44.3 ± 4.8
IIB	2	59.7 ± 15.5	7.3 ± 4.0	33.0 ± 11.5	43.9 ± 2.1
IV	1	51.6 --	5.2 --	43.2 --	43.7 --
		p[a] = 0.35	p = 0.50	p = 0.23	p = 1.00
Corr. Coeff.[b]		r = 0.45	r = -0.06	r = -0.51	r = 0.07
		p[c] = 0.15	p = 0.85	p = 0.09	p = 0.82

Risk Cat.	N	$\%T_S$ Mean S.D.	T_H/T_S Mean S.D.	%T(total) Mean S.D.	%B cells Mean S.D.
I	6	18.5 ± 3.8	2.3 ± 0.7	71.3 ± 8.6	25.7 ± 8.0
IIA	3	18.2 ± 1.8	2.4 ± 0.1	73.3 ± 9.4	25.2 ± 3.1
IIB	2	18.8 ± 4.5	2.4 ± 0.7	68.5 ± 0.1	22.6 ± 4.2
IV	1	17.0 --	2.6 --	72.1 --	27.8 --
		p[a] = 0.99	p = 0.86	p = 0.77	p = 0.82
Corr. Coeff.[b]		r = -0.02	r = 0.18	r = 0.07	r = -0.32
		p[c] = 0.96	p = 0.57	p = 0.84	p = 0.31

[a] Confidence limits for differences among risk groups.
[b] Spearman correlation coefficient for blood lead (BPb).
[c] Confidence limits for correlation with BPb.

Table 6: Cellular Data for Age 37+ Months

Risk Cat.	N	%Lymph Mean S.D.	%Mono Mean S.D.	%Gran Mean S.D.	$\%T_H$ Mean S.D.
I	32	42.6 ± 10.3	6.5 ± 2.6	51.0 ± 9.9	44.5 ± 4.4
IIA	9	40.8 ± 13.9	6.4 ± 2.2 (8)	52.3 ± 14.4 (8)	47.3 ± 8.4
IIB	6	43.4 ± 9.0	5.3 ± 1.3	51.2 ± 9.9	42.0 ± 6.3
III	6	40.5 ± 8.5	6.2 ± 1.9	53.3 ± 9.4	46.5 ± 6.7
IV	1	30.2 --	4.7 --	65.1 --	45.1 --
		p[a] = 0.74	p = 0.80	p = 0.72	p = 0.53
Corr. Coeff.[b]		r = -0.01	r = -0.21	r = 0.05	r = -0.02
		p[c] = 0.92	p = 0.13	p = 0.75	p = 0.91

Risk Cat.	N	$\%T_S$ Mean S.D.	T_H/T_S Mean S.D.	%T(total) Mean S.D.	%B cells Mean S.D.
I	32	21.2 ± 4.7	2.2 ± 0.6	76.8 ± 5.6	20.8 ± 6.4
IIA	9	17.3 ± 4.0	2.9 ± 1.2	74.9 ± 4.0	22.7 ± 6.2
IIB	6	21.5 ± 4.9	2.0 ± 0.5	72.8 ± 10.9	24.6 ± 10.4
III	6	21.8 ± 7.5	2.5 ± 1.2	77.3 ± 4.8	20.6 ± 6.4
IV	1	22.6 --	2.0 --	81.8 --	17.8 --
		p[a] = 0.24	p = 0.36	p = 0.45	p = 0.75
Corr. Coeff.[b]		r = 0.00	r = -0.03	r = -0.09	r = 0.11
		p[c] = 0.98	p = 0.84	p = 0.53	p = 0.42

[a] Confidence limits for differences among risk groups.
[b] Spearman correlation coefficient for blood lead (BPb).
[c] Confidence limits for correlation with BPb.

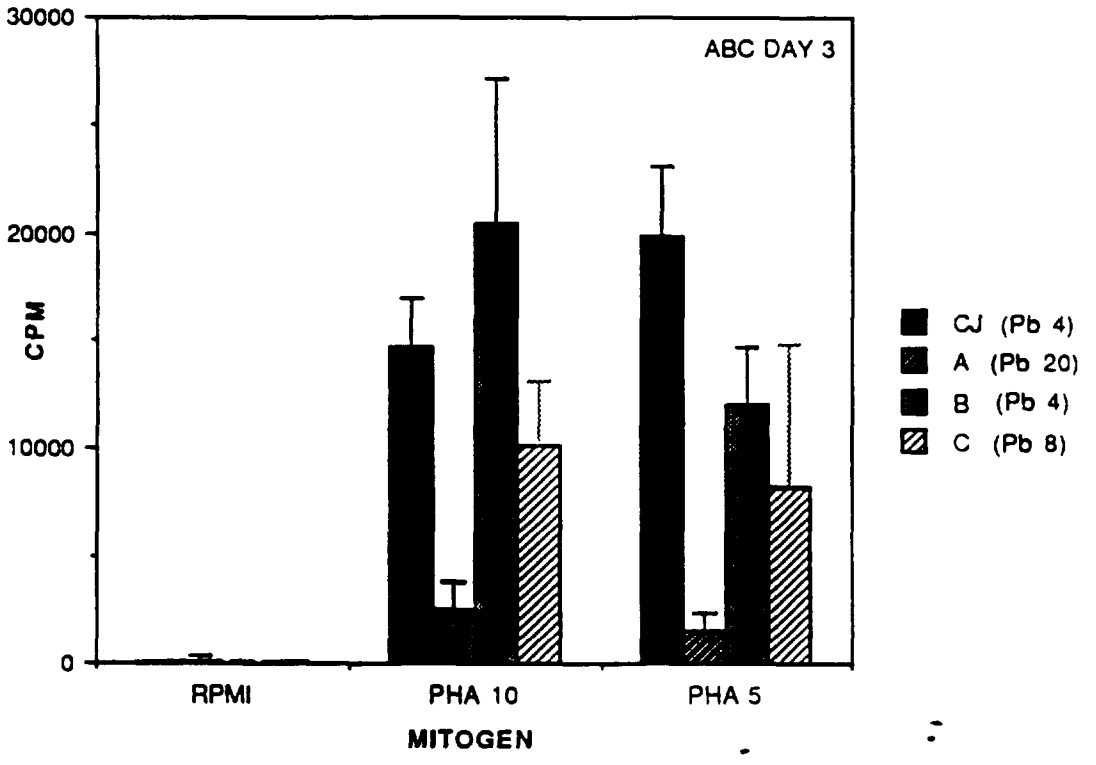

Figure 2. ABC assay Day 3.

Patients A, B, and C. Blood lead values: A = 20 μg/dl; B = 4 μg/dl; C = 8 μg/dl; CJ = 4 μg/dl. Results shown are the mean of quadruplicate samples, with standard deviation indicated for each bar. Two concentrations of PHA were used [10 μl and 5 μl/ml of a stock suspension (Difco)] and two concentrations of Con A [4 μl and 2 μl/ml; 40 μg and 20 μg/ml respectively] were used. The assay was harvested on day 3.

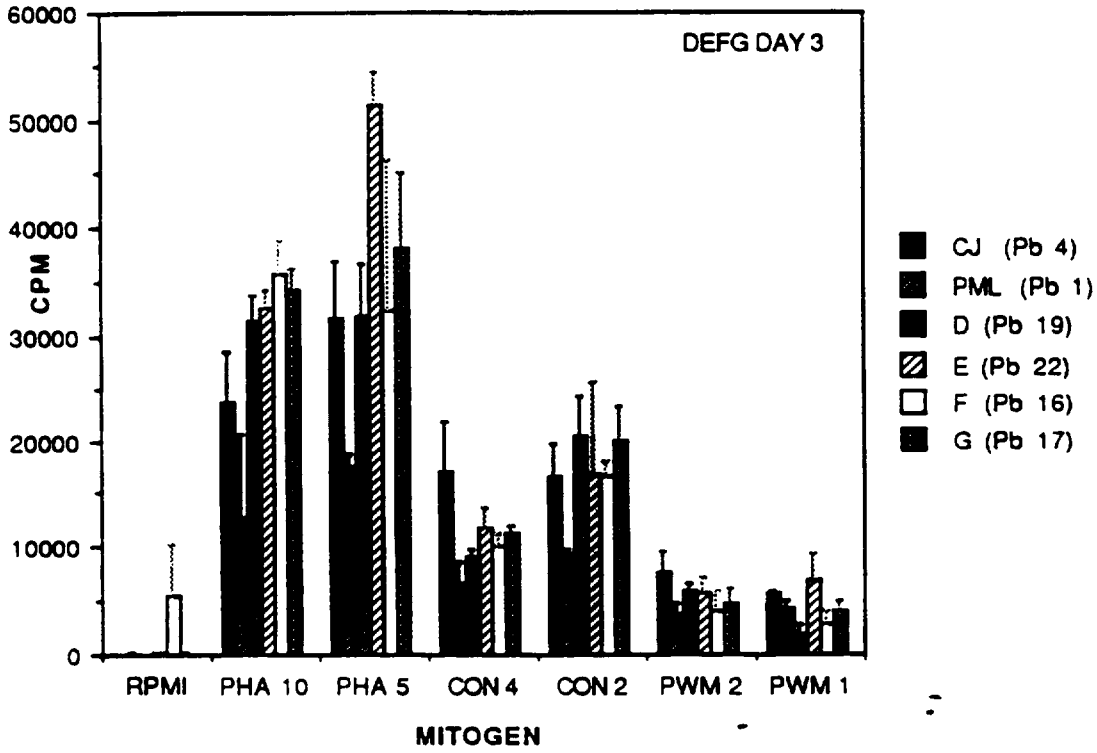

Figure 3. DEFG assay Day 3.

Patients D, E, F, and G. Blood lead values: D = 19 μg/dl; E = 22 μg/dl; F = 16 μg/dl; G = 17 μg/dl; PML = 1 μg/dl; CJ = 4 μg/dl. Results shown are the mean of quadruplicate samples, with standard deviation indicated for each bar. Two concentrations of PHA were used [10 μl and 5 μl/ml of a stock suspension (Difco)], two concentrations of Con A [4 μl and 2 μl/ml; 40 μg and 20 μg/ml respectively], and two concentrations of PWM [2 μl and 1 μl/ml of a stock suspension from Gibco] were used. The assay was harvested on day 3.

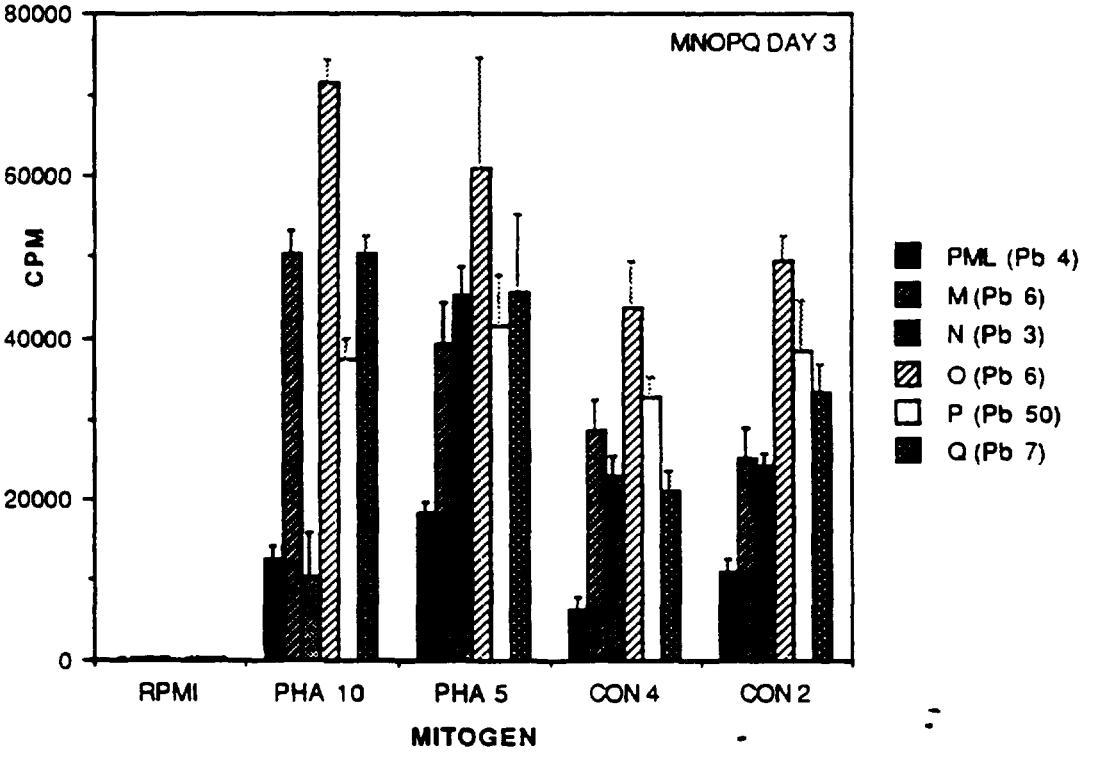

Figure 4. MNOPO assay Day 3.

Patients M, N, O, P, and Q. Blood lead values: M = 6 μg/dl; N = 3 μg/dl; O = 6 μg/dl; P = 50 μg/dl; Q = 7 μg/dl; PML = 4 μg/dl. Results shown are the mean of quadruplicate samples, with standard deviation indicated for each bar. Two concentrations of PHA were used [10 μl and 5 μl/ml of a stock suspension (Difco)] and two concentrations of Con A [4 μl and 2 μl/ml; 40 μg and 20 μg/ml respectively] were used. The assay was harvested on day 3.

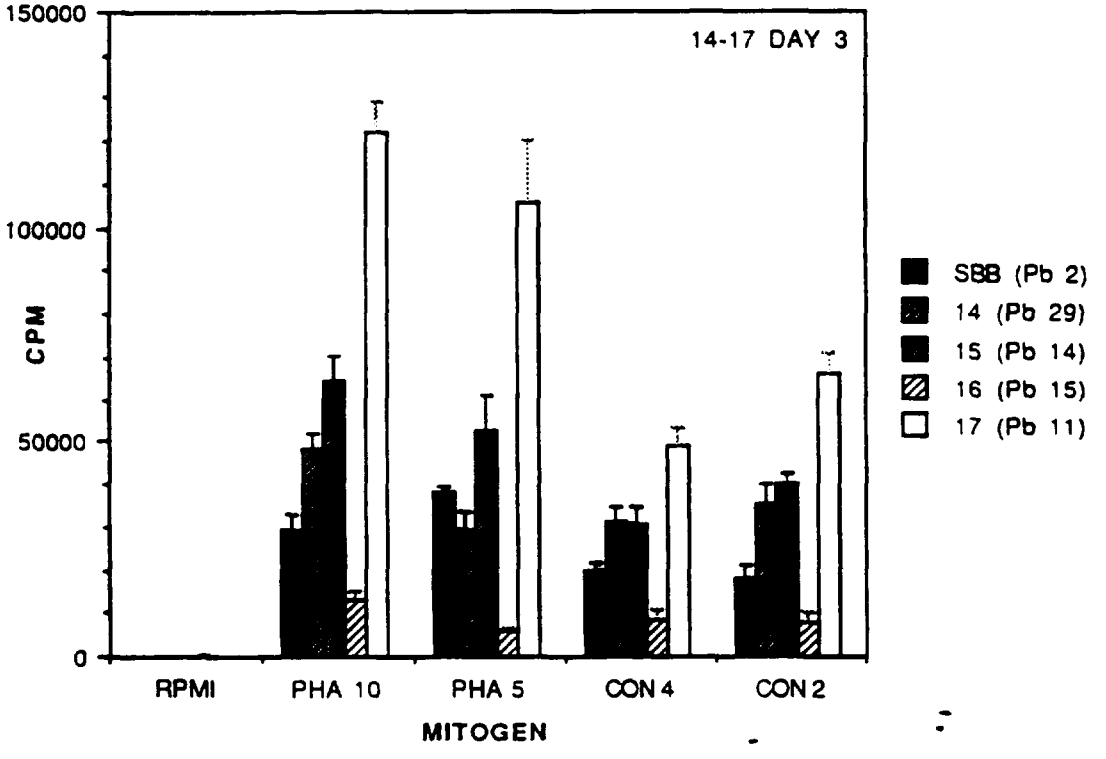

Figure 5. 14-17 assay Day 3.

Patients 14, 15, 16 and 17. Blood lead values: 14 = 29 μg/dl; 15 = 14 μg/dl; 16 = 15 μg/dl; 17 = 11 μg/dl; SBB = 2 μg/dl. Results shown are the mean of quadruplicate samples, with standard deviation indicated for each bar. Two concentrations of PHA were used [10 μl and 5 μl/ml of a stock suspension (Difco)], two concentrations of Con A [4 μl and 2 μl/ml; 40 μg and 20 μg/ml respectively], and two concentrations of PWM [2 μl and 1 μl/ml of a stock suspension from Gibco] were used. The assay was harvested on day 3.

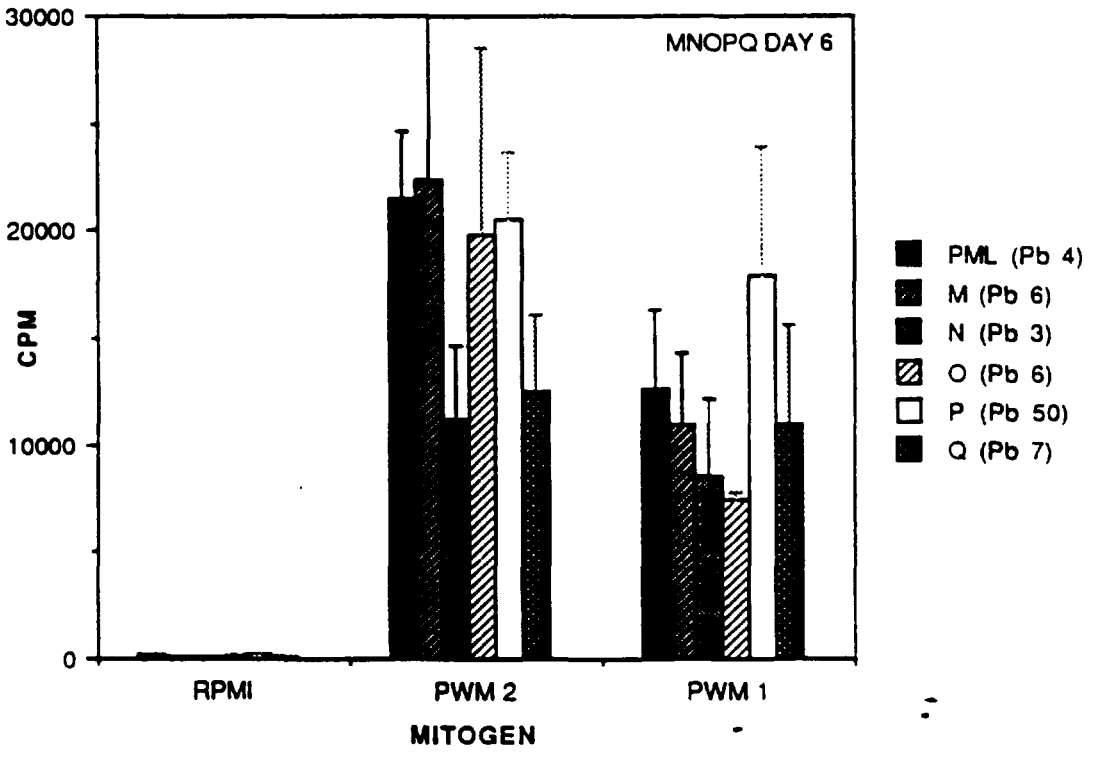

Figure 6. MNOPQ assay Day 6.

Patients M, N, O, P, and Q. Blood lead values: M = 6 μg/dl; N = 3 μg/dl; O = 6 μg/dl; P = 50 μg/dl; Q = 7 μg/dl; PML = 4 μg/dl. Results shown are the mean of quadruplicate samples, with standard deviation indicated for each bar. Two concentrations of PWM [2 μl and 1 μl/ml of a stock suspension from Gibco] were used. The assay was harvested on day 6.

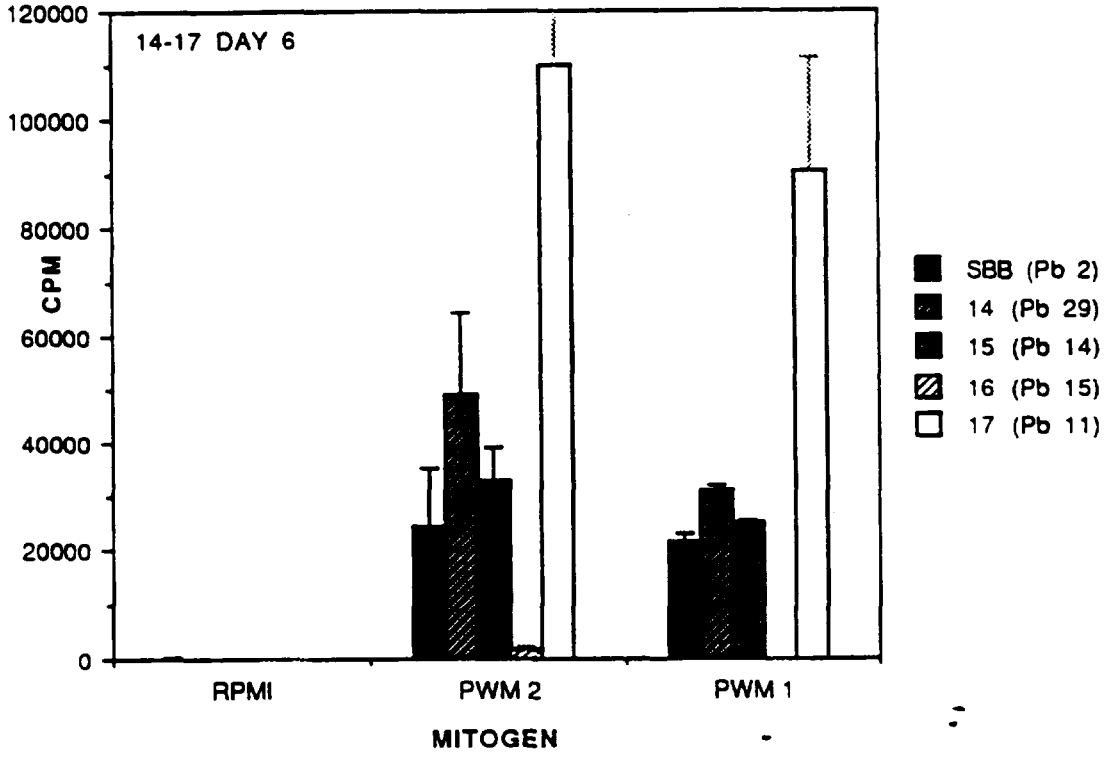

Figure 7. 14-17 assay Day 6.

Patients 14, 15, 16 and 17. Blood lead values: 14 = 29 μg/dl; 15 = 14 μg/dl; 16 = 15 μg/dl; 17 = 11 μg/dl: SBB = 2 μg/dl. Results shown are the mean of quadruplicate samples, with standard deviation indicated for each bar. Two concentrations of PWM [2 μl and 1 μl/ml of a stock suspension from Gibco] were used. The assay was harvested on day 6.

MNOPQ with the Class IV child and 14-17 with four higher risk class children. No significant correlations were found between BPb and uptake of ^{3}H-thymidine in these or other PWM stimulation assays ($p > 0.05$).

Multivariate analyses which attempt to correlate BPb and age with one of the other cellular parameters show that age always makes the most important contribution to the value of that parameter ($p < 0.05$ for all parameters; data not shown). The immune system is maturing in children of this age range, and is in a state of flux. Data comparison for immunological parameters must consider age as a contributing factor. Thus, though it is tempting to pool the high risk categories in several age groups to give a larger sample size and a higher degree of confidence, it is not appropriate with such age-dependent data. Although such regression analyses fail to show a significant contribution by BPb to the value of the parameter, this may not indicate that BPb has no effect. It shows only that in the presence of the overwhelming contribution of age, BPb adds little to the model. This reiterates the need to compare these parameters only within age groups.

The lack of statistically significant differences among the risk groups and of correlations of BPb with the various cellular parameters in the age groups is perhaps not surprising. The parameters quantitated here assess the absolute numbers of the various cell types involved in the immune response and the broad, non-specific responses of lymphocytes to mitogens. At best these represent indirect indicators of immune status. In animal studies, the best evidence for immunosuppression by lead comes from more specific assays of immune function (Koller, 1973; Cook *et al.*, 1975; Koller and Roan, 1980; Lawrence, 1981; Kerkvliet and Baecher-Steppan, 1982; Koller *et al.*, 1985). Indeed, immunosuppression by lead is suggested in this population in the accompanying paper (Lutz *et al.*, 1992a) where specific antibody titers are measured. However, the parameters reported here had not been routinely measured in a population of this age range, and represent the foundation on which future functional assays will be based. Data from control (Class I) children for these assays are sparse and represent a valuable resource for other investigators as well.

In the mitogen stimulation assays, the variation in the optimal response to these mitogens in the controls is clearly seen. Such variation has been a known deterrent to the sensitivity of this technique. With such interindividual oscillation, differences must be large to be seen. The effects of lead, if any, are most likely subtle and so may be obscured in the complex kinetics of proliferation in this hetergeneous mononulear cell preparation. Nevertheless, the previously mentioned data in animal studies, though confusing, indicated that at least a preliminary analysis of the possible effects of lead on this response was justified. In future studies, the broadly specific mitogen assays will be replaced with stimulation by specific antigens.

Attempts have been made to control for other factors which may affect these parameters. Socio-economic level is controlled through eligibility for the WIC program; nutrition is controlled through the food coupons provided by WIC (although compliance is difficult to verify). Comparisons of the various parameters between males and females show no obvious gender-related differences (data not shown). [The levels of T_s's (and the related helper/suppressor ratio) do show a difference, but this appears to be the result of an older mean age in females as compared to males (31.4 months *vs.* 27.2 months).] The possible contribution of race is difficult to discern in this demographically homogeneous population; only 13 of 193 children are black, 12/193 are mixed (black/white), 1/193 is Asian, and 3/193 are Hispanic. [There is a suggestion of a difference in % T_H's, the T_H/T_s ratio, and the %T (total), but with such small numbers it is impossible to properly weight the effects of skewing due to age.]

The presence of a smoker in the child's environment must also be considered. Active smoking has been reported to depress the immune system (Ginns *et al.*, 1982; CDC, 1990; Kusaka *et al.*, 1989). Lead is also concentrated in the cigarette filter and represents a possible source of ingested lead for small children. Analyses of these data with respect to passive smoking show that children in high risk categories due to BPb are more likely to come from a home where someone smokes. Of the 114 children who were Class I, 64 (56%) were exposed to passive smoking. In the higher risk categories, 78% (28/36) of Class IIA, 82% (14/17) of Class IIB, 92% (22/24) of Class III, and 100% (2/2) in Class IV resided in a "smoking" home. In the 130/193 children in homes where smoking occurred, the average BPb was 12.05 μg/dl. In the 63/193 "non-smoking" homes, the BPb averaged 6.95 μg/dl ($p = 0.0001$). Possible differences in the %T_s's, the T_H/T_s ratio, and the

% B cells were noted (data not shown); comparisons of children in smoking and non-smoking homes needs to be done within age groups to verify this possibility. It is difficult to isolate smoking from other closely correlated socioeconomic factors in this study population.

In summary, the data show that while two age groups showed a possible effect of lead, no significant correlation with BPb could be detected for any of these parameters within these age groupings. Mitogen stimulation assays using PHA, Con A, and PWM failed to detect differences attributable to lead amid the inherent variation in controls. Smoking in the home represents a possible confounding variable in these studies and should be further analyzed. Future (Phase II) studies will include more assays of specific immunity, such as the response to specific antigens; the addition of activation antigens such as CD25 (the IL2 Rc) to the cytofluorimetry profile; and a longitudinal study with multiple samples taken from each child over a period of months or years.

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