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Exacerbation of Soft Tissue Lesions in Lead Exposed Virus Infected Mice[1]

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Objective To investigate the effect of Lead (Pb) acetate exposure on Semliki forest virus (SFV) pathogenesis in mice. **Methods** Different doses (62.5, 125, 250 and 500 mg/Kg body weight) of Pb dissolved in normal saline were given to mice by oral intubation in a sub-acute (28 days) and sub-chronic (90 days) regimen followed by SFV infection. Morbidity, mortality, clinical symptoms, mean survival time (MST), changes in body and organ weight, accumulation of lead in soft tissues, virus titre in brain and histopathological alterations were compared between lead exposed and infected groups. **Results** Early appearance of virus symptoms, increased mortality, decreased MST, enhanced SFV titre and greater tissue damage were observed in lead exposed-SFV-infected mice. **Conclusion** Pre-exposure to lead increases the susceptibility of mice towards SFV infection. Further studies are suggested in view of the persistence of lead in the environment and the possibility of infection by microbial pathogens.

Key words: Lead acetate; Exposure; Sub-acute; Sub-chronic; SFV; Pathogenicity

INTRODUCTION

Lead (Pb) is one of the major pollutants in terms of global contamination and health impacts. It is an important non-biodegradable environmental toxicant affecting all segments of the population, especially children, industrially and occupationally exposed groups and pregnant women[1-3]. Despite several regulations and efforts by regulatory agencies to control lead pollution by limiting its industrial uses and continued emphasis on lead free gasoline consumption in India and other developing countries, low levels of lead are still present in air, water and soil, particularly near urban and industrial areas. Clinical consequences of lead toxicity, though variable, have been shown to affect almost every system including nervous, cardiovascular, hepatic, renal, haematological and immune systems. Inorganic metals like lead, cadmium and manganese have been shown to be immunosuppressive and therefore enhance the susceptibility of the host towards bacterial and viral infections often creating life threatening state[4-11].

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In humans, lead exposure has been reported to induce immunotoxic abnormalities like impaired response to mitogens[12-14] alterations in the number of lymphocytes[14,15], decreased levels of immunoglobulins[6,15,16], depression of neutrophil functions[17-19] and increased incidence of colds and influenza[5,6]. More recently, UNDEGER *et al.* [20] and SATA *et al.*[21,22] have reported alterations in human T-cell and B-cell subpopulations, NK cell, complement protein (C3, C4) concentrations and serum immunoglobulins (IgG, IgA, and IgM) and other detrimental effects of lead on the immune system.

Evaluation of the potential toxicity of lead under the influence of viral infection has been our concern and hence we have investigated the effects of subacute and subchronic oral toxicity of lead acetate in mice on the pathogenicity of Semliki forest virus (SFV). Data presented here indicate that lead intoxication caused early appearance of clinical signs, higher mortality, reduced mean survival time (MST), significantly enhanced viral titers and greater tissue damage in the lead exposed-virus infected mice compared to non-infected animals.

MATERIALS AND METHODS

Chemicals

Lead acetate used was obtained from Merck. All other chemicals and reagents used were of high purity grade.

Animals, Virus and Cells

Swiss albino mice, 8-10 gm body weight (bw) obtained from the Industrial Toxicology Research Centre breeding colony were maintained on a standard pellet diet and water *ad libitum* and were housed in environmentally controlled rooms (Temperature 25℃±2℃ and humidity 50±5%) under a 12 h dark light cycle. All procedures, maintenance and treatment of mice were in accordance with the guidelines for the care of animals by the Institutional Animal Ethics Committee.

Smithburn and Haddow strain of Semliki forest virus (SFV) was obtained from American Type Culture Collection (ATCC) and was passaged intracerebrally in Swiss mice (6-7 g). Mice showing moribund infectivity signs and typical hind limb paralysis were sacrificed and brain tissue was aseptically excised, homogenized and centrifuged at 5 000 r.p.m. for 30 minutes. Supernatant serving as stock virus was stored at -80℃.

Baby hamster kidney (BHK-21) cells used for *in vitro* viral titration were procured from the National Centre for Cell Science (NCCS), Pune. The cells were sub-cultured in minimum essential medium (MEM, Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA) and antibiotics (100 μg/mL streptomycin, 100 units of benzyl penicillin/mL and 40 μg/mL gentamycin) and maintained in MEM containing 2.5% FBS and antibiotics. Viral titration was carried out by cytopathic effect assay of SFV in BHK cell line and represented as $TCID_{50}$[23].

Treatment

To observe the effect of potential interaction of repeated doses of lead and SFV infection in sub-acute (28 days) and sub-chronic (90 days) toxicity studies, mice (8-10 g) were given different (62.5, 125, 250 and 500 mg/kg bw) doses of lead acetate (5 d/w) dissolved in normal saline by gastric intubation while the control group of animals received

equal volume of normal saline. Ten mice per group were taken for sub-acute study and 12 mice in each group for the sub-chronic study. Lead exposed animals were infected with SFV subcutaneously (sc) after completion of the sub-acute and/or sub-chronic lead exposure; the day of infection was counted as day zero (0). Animals were monitored daily in the morning and evening for clinical signs, morbidity and mortality up to 14 days. For virus titration, animals were sacrificed 14 days post infection and brain tissue samples were collected, pooled and frozen group wise for virus titre assay and also a part of brain, liver and kidney tissue was placed in 10% formalin for histopathological studies.

In vitro Virus Titration

BHK–21 cells were grown in 96 well microtitre plates (Nunc, USA) and then infected with serially (10 fold) diluted 10% (w/v) brain homogenate obtained from the lead exposed as well as control group of infected mice and incubated at 37℃ in humid atmosphere of 5% CO_2 incubator (Heraeus Hera cell model, Germany) for 48 h. The plates were later stained with 0.1% crystal violet (Sigma, USA) and examined under inverted microscope (Leica, Germany) for cytopathic effect, which was graded on the standard scale of normal to virtually complete destruction of cell monolayer.

Histopathology

To evaluate and compare the tissue lesions, sections of formalin fixed and paraffin embedded brain, liver and kidney of mice of different treatment groups at identical time points were prepared. Five-micron thick sections were then stained with hematoxylin and eosin (H & E) and examined microscopically.

Estimation of Lead in Target Organs

Lead content was estimated in brain, liver and kidney of lead exposed animals as described by Berman[24]. Briefly, the tissues were digested in an HNO_3:$HClO_4$ (6:1) mixture and slowly evaporated to dryness. The residue was dissolved in an appropriate volume of 0.1 mmol/L HNO_3 and lead concentration was measured in atomic absorption spectrophotometer (Varian 250 Plus, USA).

Statistical Analysis

The data were analyzed statistically by Student's *t*-test for the level of significance and presented as $\bar{x} \pm s$.

RESULTS

Mortality and Mean Survival Time

Lead exposure at 250 mg and 500 mg/kg bw caused 10% and 30% mortality respectively in 28 days and 58% and 83% mortality after 90 days period (Table 1). Mice surviving subacute and subchronic exposure were infected with SFV and further observed for 14 days to monitor virus induced clinical signs of morbidity and mortality. Animals treated with lead (250 and 500 mg/kg bw) for 28 days exhibited a higher mortality (22% and 42%) following SFV challenge. SFV infection to non-lead exposed (i. e. virus control mice receiving only saline) mice caused 10% mortality in 28 days study compared to no mortality observed in saline treated mice of 90-day study. The lack of mortality in the 90 days study may be

attributed to age related resistance. Symptoms of sickness like roughening of hair coat, sluggish movements, partial or total paralysis were prominent in SFV challenged lead exposed mice compared to SFV alone or lead alone group. Reduced mean survival time (MST) was observesd in all groups of virus challenged lead exposed mice irrespective of administered dose and duration of lead exposure (Table 1).

TABLE 1

Effect of Pre-Oral Exposure to Lead Acetate on SFV Infection in Mice: Sub-acute and Sub-chorinc Study

Treatment	Sub-acute Study (28 Days)		Sub-chronic Study (90 Days)	
	Total No. of Mice Survived/Total No. of Mice	Mortality (%)	Total No. of Mice Survived/Total No. of Mice	Mortality (%)
Control (Normal Saline)	10/10	0.0	12/12	0.0
Lead Acetate 62.5 mg/kg	10/10	0.0	10/12	16.66
Lead Acetate 125 mg/kg	10/10	0.0	8/12	33.33
Lead Acetate 250 mg/kg	9/10	10.0	7/12	58.32
Lead Acetate 500 mg/kg	7/10	30.0	2/12	83.33

Note. SFV Challanged 28 or 90 days after lead exposure and infected mice observed for 14 days post infection.

Virus Infection	Sub-acute Study (28 Days)			Sub-chronic Study (90 Days)		
	Total No. of Mice Survived / Total No. of Mice	Mortality (%)	MST (Days)	Total No. of Mice Survived / Total No. of Mice	Mortality (%)	MST (Days)
Control (Normal Saline) + SFV	9/10	10.0	13.82	12/12	0.0	14.0
Lead Acetate 62.5 mg/kg + SFV	9/10	10.0	12.87	9/10	10.00	13.14
Lead Acetate 125 mg/kg + SFV	9/10	10.0	12.62	7/8	12.50	11.40
Lead Acetate 250 mg/kg + SFV	7/9	22.2	11.75	5/7	28.60	9.82
Lead Acetate 500 mg/kg + SFV	4/7	42.9	9.37	ND	ND	ND

Note. MST=Mean Survival Time; ND=Not Done. (Experiments could not be continued further due to high animal mortality)

Body Weight, Organ Weight, and Residue Level

Significant reduction in body weight gain in mice along with the decrease in absolute weight of tissues was observed after 28 days and 90 days exposure of lead acetate. Marked decrease in hepatic and renal tissue weight was observed at 250 and 500 mg/kg bw dose in subacute regimen and at 125 and 250 mg/kg bw dose in 90 days study. Experiments of subchronic regimen, 500 mg/kg bw dose were discontinued due to excessive mortality.

Data presented in Table 2 show the concentration of lead in brain, liver and kidney of exposed mice. Highly significant ($P<0.001$) accumulation of Pb occurred in the tissues of 90 days exposed mice of all the groups while only liver and kidneys from the mice of 28 day exposed groups (250 mg/kg and 500 mg/kg bw) showed significant Pb accumulation.

Virus Titre

Virus titre in brain of Pb exposed SFV infected mice was found to be increased in comparison to non lead exposed virus infected (virus control) animals and the increase in

the virus titre followed enhancement related to the dose of Pb both in 28 days and 90 days exposures (Fig.1). Comparatively higher titre was observed in all doses of lead exposed and SFV infected groups of mice under sub-acute lead exposure regimen. Slightly lower titre in identical groups of subchronically exposed mice compared to mice given subacute exposure indicated age related difference between the groups.

TABLE 2

Concentration of Lead in Tissues of Mice Orally Treated With Lead Acetate

Treatment	Tissue Lead Concentration (ppm)					
	Sub-acute Study (28 Days)			Sub-chronic Study (90 Days)		
	Brain[a]	Liver	Kidney	Brain	Liver	Kidney
Control (Normal Saline)	2.65±0.53	0.63±0.04	1.99±0.54	3.69±0.26	1.18±0.16	5.76±0.68
Lead Acetate 62.5 mg/kg	2.92±0.31	1.00±0.19	3.69±0.78	6.28±0.96[b]	3.47±0.43[e]	15.02±1.55[e]
Lead Acetate 125 mg/kg	3.15±0.24	1.18±0.23	3.73±0.77	8.97±0.91[e]	4.79±0.56[d]	21.91±2.79[e]
Lead Acetate 250 mg/kg	4.16±1.04	2.22±0.23[d]	6.96±1.03[c]	10.49±0.94[e]	7.24±0.89[e]	30.75±0.37[e]
Lead Acetate 500 mg/kg	9.04±1.95[b]	4.56±1.14[e]	11.42±1.01[e]	ND	ND	ND

Note. [a]: Values represent±SE of 4-8 mice per group; [b]: $P<0.05$; [c]: $P<0.02$; [d]: $P<0.01$; [e]: $P<0.001$; ND=Not Done (Please see foot note in Table 1)

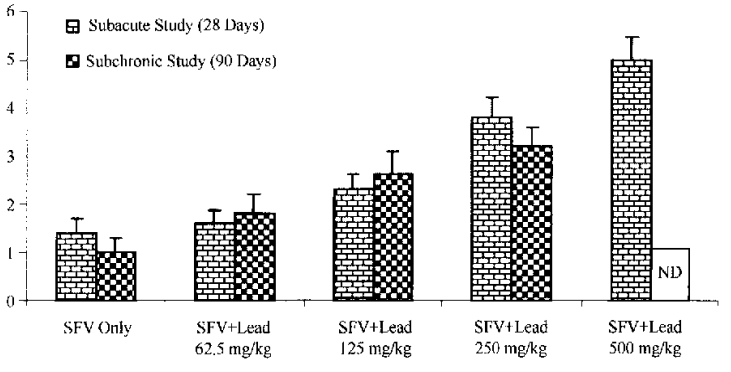

FIG. 1. Effect of oral lead exposure (subacute study 28 days and subchronic study 90 days) followed by SFV challenge on virus titre in mice brain 14-day post infection.

Histopathological Findings

Liver Following 28 day oral exposure to 62.5 mg/kg bw Pb-acetate in mice, no significant evidence of cytotoxicity in hepatocytes or inflammatory reaction in the parenchyma of liver was observed. With a higher dose of lead (125 mg/kg bw), portal congestion and varying degree of degeneration and necrosis in hepatocytes were seen. Further higher doses (250 mg and 500 mg/kg bw) caused widespread necrosis of hepatocytes and focal collection

of inflammatory cells in the portal triad areas as well as in the necrotic areas.

The liver of SFV infected mice (Virus control group of mice not exposed to Pb) at 14 days had moderate to marked congestion, varying degree of degenerative changes including necrosis of hepatocytes and inflammatory reaction comprising predominantly of centrilobular aggregations of mononuclear cells along with occasional polymorphs (Fig. 2).

However, the changes encountered in liver of mice exposed to 62.5 mg/kg bw of Pb-acetate and then infected with SFV consisted of widespread necrosis and degeneration of hepatocytes involving many lobules, some of which were infiltrated with lymphocytes and polymorphs (Fig. 3). These changes appeared more severe and widespread along with congestion and oedema with increasing dose of Pb-acetate (125 mg, 250 mg or 500 mg/kg bw).

Kidney Except for slight congestion in the cortical region, the glomerular and tubular architecture including interstitium appeared intact in mice pretreated with 62.5 mg/kg bw of Pb-acetate only. With increasing dose of Pb-acetate (125 mg, 250 mg or 500 mg/kg bw) there was dose-dependent damage both in the glomerular and tubular regions as evidenced by increased congestion and oedema, increased cellularity of glomeruli, moderate to marked degeneration and desquamation of tubular epithelium as well as focal collection of chronic inflammatory cells in the interstitium. Many casts could be seen in the lumen of various tubules.

Kidneys from mice not exposed to lead and infected with SFV demonstrated moderate congestion in the cortico-medullary region, oedema in the interstitium along with a few foci of lymphocytic cells, while tubular epithelial cells generally appeared swollen together with degeneration and necrotic changes (Fig. 4).

Mice exposed to lower doses of Pb-acetate (62.5 mg or 125 mg/kg bw) and infected with SFV, however, exhibited enhanced damage in the kidney in the form of haemorrhagic and oedematous areas, hypertrophied glomeruli having increased cellularity and widespread tubular epithelial degeneration, desquammation and necrosis. In addition, there was also widespread infiltration of chronic inflammatory cells in the interstitium (Fig. 5). With increasing doses of Pb-acetate (250 mg or 500 mg/kg bw), the kidney demonstrated more severe tubular damage along with widespread congestion and oedema.

Brain The various regions of brain of mice did not reveal any significant histopathological alteration following 28-days exposure to lowest dose of Pb-acetate (62.5 mg/kg bw) except for mild indication of glial proliferation and congestion in the white pulp of cerebellum. With increasing dose of Pb-acetate (125 mg, 250 mg or 500 mg/kg bw) there was a general dose dependent increase in the degree of congestion including meninges, cerebral cortex, thalamus, hippocampal area and cerebellum. The glial proliferation was quite prominent and neuronal damage was minimal in these animals.

SFV infected mice at 14 days showed widespread congestion of blood vessels in many parts of brain including meninges, cerebral cortex, thalamus, white matter of cerebellum with differential perivascular cuffing area and neuronal damage (Fig. 6).

Animals exposed to Pb-acetate (62.5 mg or 125 mg/kg bw) and then infected with SFV exhibited more damage in the brain of mice as evidenced by widespread congestion, focal necrotic areas involving neurons and glial cells, vacuolation of myelin fibers in the different parts of the brain including many large perivascular cuffing areas (Fig. 7). More severe histopathologic changes were encountered in various parts of brain of mice exposed to higher doses of lead (250 mg or 500 mg/kg bw) and subsequent SFV infection.

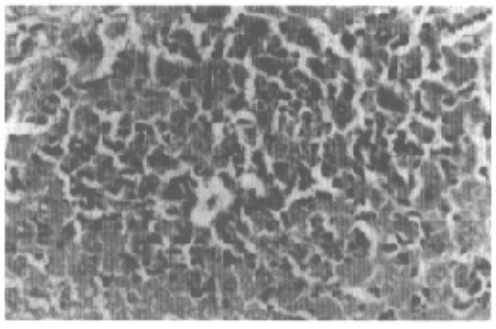

FIG. 2. Liver of saline treated mouse (28 days) after challenge with SFV, at 14 days post infection, showing centrilobular infiltration of mononuclears and necrosis of hepatocytes. H and E, ×276.

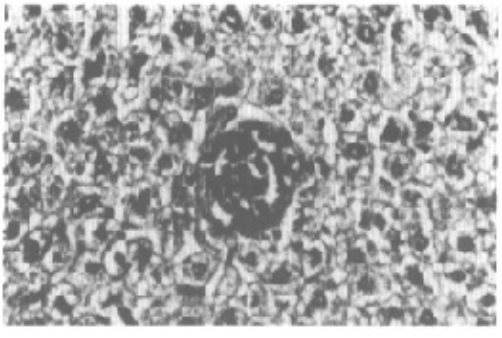

FIG. 3. Liver of mouse treated with (62.5 mg/kg) of lead acetate (28 days) and challenged with SFV at 14 days post infection, showing widespread necrosis of hepatocytes and focal infiltration of mononuclears and polymorphs. H and E, ×276.

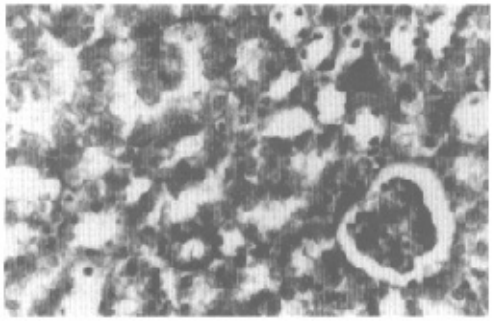

FIG. 4. Kidney of saline treated mouse (28 days) after challenge with SFV, at 14 days post infection, showing swollen tubular epithelial cells, along with moderate degenerative changes. H and E, ×276.

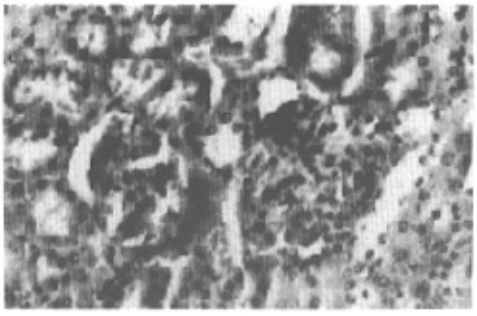

FIG. 5. Kidney of mouse treated with (62.5 mg/kg) of lead acetate (28 days) and challenged with SFV at 14 days post infection, showing hypertrophied glomerulus along with marked tubular degeneration and necrotic changes. H and E, ×276.

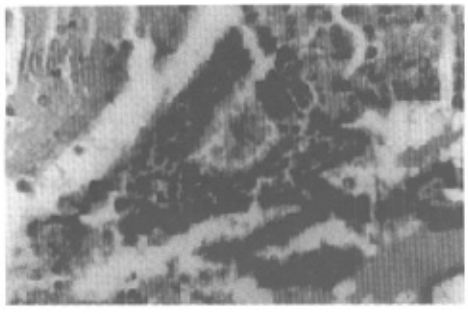

FIG. 6. Brain of saline treated mouse (28 days) after challenge with SFV, at 14 days post infection, showing typical perivascular cuffing. H and E, ×276.

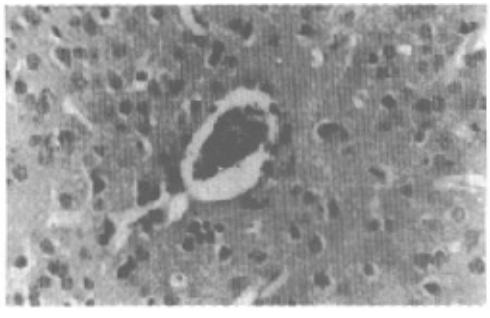

FIG. 7. Brain of mouse treated with (62.5 mg/kg) of lead acetate (28 days) and challenged with SFV at 14 days post infection, showing area of perivascular cuffing, and widespread necrosis of neuronal and glial cells. H and E, ×276.

DISCUSSION

Recent studies have shown that exposure to lead, besides causing direct toxicity,

suppresses the immune system resulting into the increased susceptibility of the host towards infectious pathogens[25-28]. In the present study, we have attempted to investigate the effects of lead exposure in mice and the susceptibility of lead exposed mice against SFV infection. Our results demonstrating early appearance of SFV symptoms, increased mortality and decreased MST (Table 1) along with elevated virus titre (Fig. 1) and enhanced pathological effects (Figs. 2-7) in lead exposed mice are indicative of decreased resistance towards SFV infection. These observations are in agreement with earlier findings reported with Langat virus, encephalomyocarditis, rauscher leukemia and pseudorabies virus[29-32]. Decrease in antibody synthesis or response[8,31] and inhibition of interferon production[30, 33, 34] appeared likely to be associated with increased mortality of lead exposed and virus infected animals.

Lead has been reported to be widely but unevenly distributed in all the soft tissues of the body following exposure, and the kidney is one of the major target organs of lead toxicity[35]; our results have shown highly significant ($P<0.001$) concentration of lead in renal and hepatic tissue of mice (Table 2). The renal involvement in lead toxicity is manifested as nephropathy that may ultimately lead to kidney failure[36]. Enhanced renal damage was found in mice with SFV infection after low doses of lead exposures in the present study. Similarly, enhanced pathological lesions were noticed in the liver and brain of SFV infected mice receiving lower exposures to lead acetate. Our observations indicating much higher severity of pathological lesions in low lead exposed and SFV infected animals suggest the need for further studies on the clinical consequences of such (lead, host, virus) interactions, specially in the context of prolonged exposure of the human population to low doses of lead.

Alterations in the blood brain barrier integrity contributing to increased permeability have also recently been highlighted using Sindbis virus and irreversible and reversible cholinesterase inhibitors in mouse model[37]. Such a mechanism might have also augmented early development of the lesions with the interaction of lead and SFV in the present study. The results of this study however, do not indicate the effects of either of the agents on the innate immunity, which plays an essential role in the regulation of all aspects of adaptive immunity. How such foreign chemicals influence the recognition and functioning of non-clonal receptors (pattern recognition receptors) of the innate immune system, which appears to be an essential antecedent to the development of initiation of an adaptive immune response subsequent to infectious agents, requires more researches.

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