Numerous studies on lead (Pb) neurotoxicity have indicated this metal to be a dangerous toxin, particularly during developmental stages of higher organisms. Astrocytes are responsible for sequestration of this metal in brain tissue. Activation of astroglia may often lead to loss of the buffering function and contribute to pathological processes. This phenomenon is accompanied by death of neuronal cells and may be connected with inflammatory events arising from the production of a wide range of cytokines and chemokines. The effects of prolonged exposure to Pb upon glial activation are examined in immature rats to investigate this potential proinflammatory effect. When analyzed at the protein level, glial activation is observed after Pb exposure, as reflected by the increased level of glial fibrillary acidic protein and S-100β proteins in all parts of the brain examined. These changes are associated with elevation of proinflammatory cytokines. Production of interleukin (IL)-1β and tumor necrosis factor-α is observed in hippocampus, and production of IL-6 is seen in forebrain. The expression of fractalkine is observed in both hippocampus and forebrain but inconsiderably in the cerebellum. In parallel with cytokine expression, signs of synaptic damage in hippocampus are seen after Pb exposure, as indicated by decreased levels of the axonal markers synapsin I and synaptophysin. Obtained results indicate chronic glial activation with coexisting inflammatory and neurodegenerative features as a new mechanism of Pb neurotoxicity in immature rat brain.

Key Words: Pb neurotoxicity; cytokines; neuroinflammation; S-100β; synapsin I; synaptophysin.

Inflammation-Like Glial Response in Lead-Exposed Immature Rat Brain

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Received July 21, 2006; accepted September 29, 2006

Lead (Pb), is a ubiquitous environmental pollutant. Excessive exposure to Pb in human populations still persists, despite efforts to reduce Pb levels in the ecosystem. Toxic effects of this metal are principally manifested in the central nervous system (CNS). Destruction of the blood-brain barrier (BBB), which leads to edema, loss of neurons, and gliosis, has been reported. A broad range of studies have revealed that pre- and early postnatal exposure to even low doses of Pb is extremely dangerous and results in a variety of neurological disturbances (Canfield et al., 2003; Mendola et al., 2002; Tong et al., 2000).

Although there is a long history of research on Pb neurotoxicity, proinflammatory effects of Pb in brain have yet to be investigated despite the fact that such effects have been observed in several other tissues and organs. The proliferation of T lymphocytes under Pb action has been observed in vitro (McCabe et al., 2001; Razani-Boroujerdi et al., 1999). It has also been shown that treatment of young rats with Pb results in overexpression of interleukin (IL)-10, IL-12 and increases in numbers of monocytes and T lymphocytes in spleen and thymus (Bunn et al., 2001). The production of autoantibodies against myelin basic protein and glial fibrillary acidic protein (GFAP) after Pb exposure has been reported elsewhere (El-Fawal et al., 1999; Waterman et al., 1994). This raises the possibility that Pb may affect immune processes in brain.

Several studies have shown that astroglia can accumulate and store Pb (Holtzman et al., 1984; Tiffany-Castiglioni et al., 1986), suggesting that astroglia may function as a “Pb sink” in the brain, possibly because these cells are more resistant to the toxic effect of metals than other cell types such as neurons (Tiffany-Castiglioni, 1993). Glial cells are the predominant CNS-antigen presenting cells involved in inflammatory processes and the interaction between glia and neurons plays a vital role in the function of the brain. Under many pathological conditions, activation of glial cells is observed, which results in increased production of many proteins, including S-100β. A number of studies have implicated this protein in progression of a pathologic, glial-mediated proinflammatory state in the CNS (Craft et al., 2005; Griffin et al., 1998; Mrak et al., 1995). Both astroglia and microglia in activated form may generate and/or maintain the inflammatory reaction in brain by producing cytokines. Cytokines are key polypeptide mediators of inflammation and include interleukins, chemokines, tumor necrosis factors, interferons, and neurotrophins. Under physiological conditions, the expression levels of cytokines in the CNS are very low or undetectable due to tight expression control. However, the expression of various cytokines tends to increase following the occurrence of pathological stimuli such as injury, infection, autoimmune...
disease, or exposure to toxic agents (Gonzales-Scarano and Baltuch, 1999; Oprica et al., 2003). Several cytokines are implicated in neuronal cell damage, while others play a neuroprotective role. It is postulated that inflammatory factors released by glia such as tumor necrosis factor (TNF)-α, IL-1β, and IL-6 contribute to the destructive processes resulting in neuronal cell death (Chao et al., 1995; Liu et al., 2000; McGeer and McGeer, 1999).

Evidence has been provided over the past few years demonstrating an important role for inflammatory processes in the pathogenesis of many forms of neurodegeneration. The unquestionable contributions of inflammatory processes to the progression of diseases such as Alzheimer’s, Parkinson’s, and multiple sclerosis have been documented (Gonzales-Scarano and Baltuch, 1999; Marx et al., 1999; McGeer et al., 1993; Mrak and Griffin, 2001).

Although earlier studies have indicated that Pb potentiates the cytotoxic effect of proinflammatory cytokines in glial cells in vitro (Lahat et al., 2002) and induces expression of TNF-α in glioma cells (Cheng et al., 2004), there is still not enough evidence in support of a proneuroinflammatory effect in vivo.

Pb is a dangerous neurodevelopmental toxicant, which is sequestered in astrocytes which are involved in neuroinflammatory events in brain. In this work we subject immature rats to prolonged Pb exposure and determine the degree of astrogliosis activation, neuroinflammation, and synaptic degeneration in the rodent brain. Attention is focused on forebrain cortex, hippocampus, and cerebellum as these regions have been reported to be sensitive to neurodevelopmental effects of Pb (Collins et al., 1982; Schneider et al., 2005).

MATERIALS AND METHODS

Animals. All procedures involving animals were carried out in strict accordance with the international standards of animal care guidelines and were approved by the local Care of Experimental Animals Committee. Four pregnant Wistar females (supplied by Medical Research Centre, Poland) were caged individually with unlimited access to water and a standard laboratory diet R-Z Wistar females (supplied by Medical Research Centre, Poland) were caged individually with unlimited access to water and a standard laboratory diet and secondary antibodies: peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (1:3,000), and 1:1,000, respectively. After washing in PBS-T, membranes were incubated for 30 min at room temperature with shaking (5 min per wash). The working solution of streptavidin-horseradish peroxidase-conjugated streptavidin was added to each membrane and incubated at room temperature for 2 h. Detection buffer A and B provided in the kit were mixed, pipetted onto the membranes, and incubated for 1 min. Signals were detected directly from the membrane using a chemiluminescence imaging system. Comparison of the signals with the table included in the kit enables identification of released cytokines.

Analysis of glial and neuronal markers by Western blotting. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described by Laemmli (1976), and then electrotransferred to nitrocellulose membranes. The membranes were incubated in phosphate-buffered saline (PBS) (80mM Na2HPO4, 20mM NaH2PO4, 100mM NaCl; pH 7.5) containing 0.05% Tween 20 (PBS-T) and 5% milk powder for 1.5 h at room temperature. The membranes were then washed in PBS-T and incubated for 1.5 h at room temperature or overnight at 4°C in PBS-T containing 5% milk powder and primary antibody (all purchased from Sigma): polyclonal anti-synapsin I, monoclonal anti-synaptophysin (clone SVP-38), or monoclonal anti-S100 (β-subunit; clone SH-B1) and monoclonal anti-GFAP in dilution 1:200, 1:400, 1:500, and 1:1,000, respectively. After washing in PBS-T, membranes were incubated for 30 min at room temperature in PBS-T containing 5% milk powder and secondary antibodies: peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) for anti-synapsin (1:5,000) and peroxidase-conjugated anti-mouse IgG for anti-synaptophysin, anti-S100β, and anti-GFAP (1:5,000). The membranes were then washed in PBS-T and cross-reacted antibodies were detected using a chemiluminescence system (ECL Western Blotting System, Amersham Bioscences, Little Chalfont, UK).

Protein assay. Protein concentration in homogenates was measured according to the method of Lowry et al. (1951) using bovine albumin as a standard.

Determination of Pb content. Blood and brain Pb levels were estimated by atomic absorption spectrophotometry with a graphite furnace (AA Scan 1 Thermos Jarrell Ash). A certified reference solution of Pb (Merck, Darmstadt, Germany) was used to generate the standard curve.

Statistical analysis. The results are expressed as mean ± SD from four experiments. Intergroup comparisons were made using the one-way analysis of variance (ANOVA) followed by post hoc Tukey test. p < 0.05 was considered significant. In the case of Pb measurements, the Student’s t-test was used to compare differences between control and exposed groups.

RESULTS

Animals in the Applied Model of Pb Toxicity

The Pb regimen used in the present experiments caused elevation of Pb in blood to the range of average concentrations characteristic of prolonged Pb exposure. The mean blood Pb level (± SD) of the control group was 3.3 ± 0.9 µg/dl while that of the exposed group was 30.8 ± 8.5 µg/dl. Differences were
statistically significant at the $p$ level $< 0.05$ ($n = 3$; Student’s $t$-test). The levels of Pb in brain homogenates obtained from control animals were below the detection limit for the applied method ($<0.002 \mu g/g$) and in Pb-treated group the mean was $0.35 \pm 0.09 \mu g/g$ ($n = 3$).

The body weights of the rats were recorded daily but we did not observe significant changes between both groups during the course of the study. There were no incidents of seizures or death before the end of the study.

**Astrocytic Activation in Immature Pb-Exposed Rat Brains**

One of the most common features of astrocytes is their reaction to CNS damage with reactive gliosis, which is associated with morphological changes inside the cell and an increased synthesis of many proteins (Norenberg, 1996; Norton et al., 1992). Classically, GFAP is used as a marker of astrocyte activation. This astrocytic response is also induced by many neurotoxic agents (O’Callaghan, 1999), including Pb (Van den Berg et al., 1996). Western blotting experiments were conducted to evaluate changes in GFAP content in brain homogenates. Densitometric analysis of bands indicated increased expression of GFAP protein after Pb treatment in all parts of brain examined (Fig. 1). The differences between treated and nontreated animals were statistically significant ($p < 0.05$).

Another specific marker of astroglial cell is S-100 protein, the enhanced expression of which is implicated in neuronal cell dysfunction and may be used as a marker of neurodegenerative changes.

We noted increased S-100β immunoreactivity of bands in homogenates obtained from Pb-treated rats compared to the respective controls (Fig. 2), with the most intense enhancement observed in the hippocampus. The differences were statistically significant at the $p$ level of 0.05 in all brain regions, and reached about 40, 25, and 15% in hippocampus, forebrain, and cerebellum, respectively.

**Cytokines Released in Pb-Exposed Immature Rat Brain**

The Ray Bio Rat Cytokine Antibody Array was used to detect the profile of released cytokines. RayBiotech’s approach has several advantages over traditionally used enzyme-linked immunosorbent assay. It can simultaneously detect many cytokines and provides a more accurate reflection of active cytokine levels because it only detects secreted cytokines, without the need for an amplification step. The profile of cytokines secreted in brain of immature rats after prolonged Pb exposure was defined using “a map” of cytokine antibodies on membranes provided with the kit. In control animals, cytokines were not detected in any brain region evaluated. Thus, only one control membrane was shown on Figure 3a. In the case of Pb-exposed rats, cytokines were detected in hippocampus and...
forebrain (Fig. 3b and 3c) but not in the cerebellum (Fig. 3d). The profiles of secreted cytokines were different in hippocampus and forebrain cortex. In the hippocampus, the proinflammatory cytokines IL-1β and TNF-α were observed, while in the forebrain, IL-6 was observed. IL-1β is one of the most important cytokines involved in the initiation of early stages of inflammation and in regulation of inflammatory response. No cytokines were observed in the tissues of the control animals under the same conditions.

Interestingly, the chemokine fractalkine was also noted in all examined regions with the lowest immunoreactivity in cerebellum. This chemokine is released by both astrocytes and microglia, when stimulated with TNF-α and interferon-γ (Yoshida et al., 2001).

**Synaptic Degeneration in Brain of Immature Pb-Exposed Rats**

Given the significant increases in expression of astrocytic S-100β and proinflammatory cytokines following prolonged Pb exposure, it was important to investigate the neuronal response under these conditions. Biochemical measurements of these two presynaptic proteins were performed.

Synaptophysin is a 38-kDa integral membrane glycoprotein found in presynaptic vesicles in neuron and neuroendocrine cells. Synapsins are neuron-specific phosphoproteins associated with small synaptic vesicles and have long been implicated in the regulation of neurotransmitter release at synapses (Chin et al., 1995). As documented in numerous studies, synapsin I and synaptophysin are probably the most specific markers of synapses throughout the central and peripheral nervous system, and the expression of synapsin I is also a precise indicator of synapse formation (Moore and Bernstein, 1989; Stone et al., 1994). A significant reduction in the expression of the two neuronal markers was observed predominantly in the hippocampus. The intensity differences between the control bands and the bands of Pb-treated rats reached 30 and 35% for synaptophysin (Fig. 4) and synapsin I (Fig. 5), respectively. In cerebellum the differences, although observed, were statistically insignificant in the case of both proteins. In forebrain there were no changes between groups. The decrease in relative levels of synaptically bound proteins leads us to suggest that axonal destruction takes place in the region of hippocampus of immature rat brain exposed to Pb.
DISCUSSION

Toxic Pb insult in the early period of development may impair both glial and neuronal function (Stoltenburg-Didinger et al., 1996; Zawia and Harry, 1996). The most important function of astroglial cells is maintenance of the homeostatic environment for proper functioning of neurons. During pathological insults, including toxic conditions, glial cells undergo rapid changes which have been described as reactive gliosis. One of the most important features connected with this phenomenon is the increased expression of two glial markers, GFAP and S-100β protein. Overexpression of both of these proteins in response to neuronal damage has been observed (Griffin et al., 1998). In the present study concerning Pb toxicity in immature rat brain, we demonstrate that activation of astroglial cells occurs as evidenced by elevation of these two astrocyte-specific proteins.

When secreted from astrocytes S-100β plays both beneficial and detrimental functions (Van Eldik and Wainwright, 2003). At low physiological concentrations, it acts as a neurotrophic factor during neurodevelopment (Donato, 2001). Under more extreme pathological concentrations, it may act as a proinflammatory cytokine and contribute to neuroinflammatory reactions and neuronal dysfunction (Koppal et al., 2001; Li et al., 2000).

The major findings of the present study include demonstration of the occurrence of increased cytokine production and evidence of axonal damage accompanying astrocytic activation in Pb-exposed immature rat brain. The observed changes are rather mild in nature, suggesting that the chronic neuroinflammatory state develops slowly and predominantly occurs in the hippocampus. Although a recent study has demonstrated immunomodulatory effects of Pb, until now there was no evidence that Pb causes proinflammatory effects in brain in vivo. Pb was shown to have the ability to stimulate lymphocyte proliferation in lymph nodes of exposed mice and provide adjuvant signals to enhance immune response to unrelated antigens (Carey et al., 2006). Increased production of cytokines such IL-4, IL-6, and IL-10 in human mononuclear blood cells was observed in vitro, although the secretion of proinflammatory cytokines was not affected under these conditions (Hemdan et al., 2005).

Glia play a role in local inflammatory processes by responding and producing cytokines such as IL-1β, IL-6, and TNF-α (Zhao and Schwartz, 1998). There is increasing evidence that neuroinflammation driven by glial activation contributes to the pathogenesis and progression of many disorders. In Alzheimer's disease, inflammatory and oxidative stress molecules produced by chronically activated glia lead to damage of neurons (Griffin et al., 1998). S-100β transgenic mice express enhanced susceptibility to neuroinflammation and neuronal dysfunction after infusion of human β-amyloid (Craft et al., 2005). The current study, in conjunction with the above-mentioned data, indicate that, under conditions of Pb toxicity, S-100β overproduced by activated glia may act as a proinflammatory trigger, further contributing to neuronal dysfunction as evidenced by decreased expression of axonal markers.

However, another scenario is also possible where activation of immune cells by Pb in the periphery leads to leukocyte infiltration and subsequent activation of micro- and astroglia. This cannot be excluded, especially in the view of evidence for Pb-induced destruction of cerebral microvessels and increased BBB permeability (Strzynska et al., 1997; Sundstrom et al., 1985). Immaturity of the BBB is undoubtedly the additional factor that facilitates dysregulation of the BBB integrity in our experimental rats. A degradation of its protective effect enables blood-derived mononuclear phagocytes to easily penetrate the nervous tissue.

As demonstrated, infiltration of leukocytes and activation of brain endothelial cells, microglia, and astrocytes are early events in pathological states such as ischemic insult (Struzyńska et al., 1998). Whether, and to what extent, the activation of microglia occurs under conditions of applied Pb toxicity warrants further detailed investigations. The results of the present study give only indirect evidence provided by the observation of elevated expression of cytokines. The existence of a cross-talk between microglia and astrocytes was demonstrated, at least in vivo. It is reasonable to speculate that such an interaction also occurs in vivo and overexpressed S100β protein may represent a critical intercellular signal.

Microglia represent a source of many cytokines and thus may exert a strong regulatory influence on other brain cells, including astrocytes. One of the most potent inducers of reactive astrogliosis is IL-1β (Herx and Yong, 2001) produced both by activated microglial cells and by astrocytes (Giulian et al., 1986). IL-1β is a specific cytokine involved in intercellular communication between glial cells in injured brain and plays a key role in the regulation of inflammatory processes. Thus, enhanced levels of this cytokine observed after prolonged Pb treatment signal the induction of mechanisms leading to the inflammatory cascade and indicate potential proinflammatory action. Interestingly, the hippocampus is the region where IL-1β and TNF-α, another proinflammatory cytokine, were observed in parallel with signs of axonal degeneration as evidenced by decreased expression of synapsin I and synaptophysin. These cytokines are reported to be potent mediators of neurodegeneration under other pathological conditions. Upregulated in response to experimentally induced ischemia (Touzani et al., 2002), these cytokines exacerbate ischemic and excitotoxic brain damage (Lawrence et al., 1998).

The immunoreactivities of synaptophysin and synapsin I were significantly reduced, particularly in hippocampus, and were well correlated with the release of proinflammatory cytokines. Pathologically, the reduction in synaptic protein immunoreactivity was observed during acute experimental autoimmune encephalomyelitis (EAE) episodes and correlated well with inflammatory cell infiltration at different EAE stages (Zhu et al., 2003). Also, in Alzheimer’s disease and other
dementia-related disorders there is a significant decrease of expression of synaptophysin in the presynaptic vesicles due to perinuclear loss. As hypothesized, loss of immunoreactivity for the presynaptic vesicle marker proteins synaptophysin and synapsin may contribute to the progression of memory decline seen in patients with Alzheimer’s disease (Dawson et al., 1999).

Interestingly, when analyzed at the protein level using RayBio, we found that the expression level of fractalkine was enhanced especially in hippocampus and forebrain. Fractalkine (CX3CL1) is a chemokine which is highly expressed in neurons and astrocytes while its receptor (CX3CR1) was shown to be present on neurons and microglial cells. Additionally, fractalkine is a principal chemokine released from the cultured neurons to promote chemotaxis of microglia upon excitotoxicity (Chapman et al., 2000). In the present study, we surmise that fractalkine may play a role as a chemoattractant for macrophages and may represent an early event in the inflammatory response to neural injury under conditions of Pb neurotoxicity. However, a neuroprotective role of fractalkine was also reported. Neuron-derived fractalkine suppresses cytokine-induced neuronal cell death by reducing the production of TNF-α and other proinflammatory cytokines and nitric oxide by activated microglial cells (Mizuno et al., 2003; Zujovic et al., 2000). The presence of fractalkine in hippocampus and forebrain after exposure to Pb may indicate its regulatory and neuroprotective role in these regions, where other proinflammatory cytokines were simultaneously noticed. In the view of above-mentioned data, our results leave an open question regarding the role of the fractalkine released in Pb-treated rats. The role of this chemokine may be either chemotactic and proinflammatory in nature or protective regulatory and neuroprotective role in these regions, where astrocytes and microglia.

In conclusion, under subacute Pb exposure during postnatal maturation, chronic glial activation is observed with coexisting inflammatory and neurodegenerative features that are excessively expressed in hippocampus of exposed immature rat brain. These observations may indicate a new mechanism by which Pb induces neuropathological processes under conditions of prolonged exposure.

ACKNOWLEDGMENTS

This study was mainly supported by grant no. 2 P06F 01326 from the polish Ministry of Scientific Research and Information Technology and partially by a statutory grant to the Medical Research Centre.

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