Dose dependent effects of chronic exposure to toluene on neuronal and glial cell marker proteins in the central nervous system of rats

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Abstract

The dose dependent effects of chronic exposure to toluene on the neuronal marker proteins (γ -enolase, calbindin-D28k) and glial cell marker proteins (α -enolase, creatine kinase-B, and β -S100 protein) were investigated in the central nervous system (CNS) of rats. Three groups of animals were exposed to 100 ppm, 300 ppm, or 1000 ppm toluene vapour eight hours a day, six days a week for 16 weeks. The contents of the marker substances were determined with enzyme immunoassays. A significant increase in the three glial cell marker proteins was noted in the cerebellum after exposure to 100 ppm toluene; a more pronounced increase occurred at the higher toluene concentrations. β -S100 protein also exhibited a dose dependent increase in the brainstem and spinal cord. On the other hand, the two neuronal cell markers did not show a quantitative decrease in the CNS. This means that the development of gliosis, rather than neurone death, is induced by chronic exposure to toluene. The significant biochemical changes induced around the threshold limit value and the concentration dependent alterations suggest that these nerve specific marker proteins may be used to evaluate solvent related damage to the CNS.

Toluene is one of the most widely used aromatic solvents in industry, and it has a high potential for

abuse. Although a number of case reports and epidemiological surveys indicate that toluene exerts its main toxic effects on the central nervous system (CNS),¹ the toxic mechanism, the early and subclinical response to chronic low level exposure, and the dose effect relations are poorly defined. The evaluation of solvent related CNS toxicity is difficult due to the lack of sensitive and specific indices to detect the early and selective lesions of nervous tissue. Recently, several studies have been conducted to determine the effects of toluene on the composition of brain lipid,² protein phosphorylation,³ monoamine metabolism,⁴ and behaviour of neurotransmitters.⁵ The neurochemical methods used, however, generally did not identify the specific types or classes of neuronal cells that are affected by neurotoxic agents. Recent progress in neurochemistry and immunohistology has confirmed that some isoenzymes or isoproteins are specifically distributed in cells of the CNS. Neurone specific γ -enolase, the enolase isozyme containing the γ -subunit, is localised in the cytoplasm of neurones, whereas α -enolase, which consists of the α -subunit, is primarily localised in glial cells in the CNS.⁶ On the other hand, calbindin-D28k and β -S100 protein belong to a family of calcium binding proteins, but have different cellular localisation in the CNS. Calbindin-D28k is localised in the cytoplasm of neurones whereas β -S100 protein is found in astroglia.^{7,8} Also, creatine kinase-B, a brain type glycolytic enzyme with the β -subunit of CK isozyme, is also known to be located primarily in glial cells.⁹ Highly sensitive immunoassay systems for determining these specific marker proteins have been developed with detection limits in the picogram range.¹⁰

The specific cellular distribution of these proteins in the nervous system and the establishment of sensitive immunoassay systems for them have made it possible to investigate the biochemical basis of solvent related neurotoxicity. In our previous study, we found that inhalation of toluene for two weeks could induce dose dependent changes in these

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neuronal and glial cell marker proteins in separate rat brain regions.¹¹ In the present study, we focused our attention on the dose dependent effects of chronic exposure to toluene for 16 weeks. We attempted to clarify the biochemical response pattern in different types of nerve cells and different regions of the CNS after prolonged toluene treatment.

Materials and methods

ANIMALS AND EXPOSURE TO SOLVENTS

Thirty two specific pathogen free male Wistar rats (about 300 g body weight; Shizuoka Laboratory Animal Centre) were randomly divided into four equal groups, and were acclimatised to the animal facility for one week before initial exposure. One group was exposed to 100 ppm, one to 300 ppm, and one to 1000 ppm toluene vapour (99% pure, Katayama Chemical Co) in stainless steel inhalation chambers (80 × 80 × 58 cm). Exposure was for eight hours a day, six days a week, for 16 weeks. The control group was housed in an identical chamber ventilated with fresh air. The environment was kept on a 12 hour light dark cycle (lights on at 900 and off at 2100), and held at 20–21°C and 57–60% humidity. All animals had free access to food pellets and water during the test period.

The inhalation exposure system used in this study was the same as that described in our previous reports.^{11,12} The amount of solvent injected into the evaporators was automatically controlled by a computer system that constantly monitored the information on chamber concentration as supplied by a gas chromatograph (Shimadzu GC-8A). The long term stability of the system allowed the concentration of solvent to be kept within 5% of the target concentration. The time weighted average concentrations of toluene during the whole exposure period in the three treatment groups were 100 ppm (standard deviation (SD) 2 ppm), 302 ppm (SD 6 ppm), and 1003 ppm (SD 24 ppm).

TISSUE PREPARATIONS

Body weight gain of the animals was measured once every two weeks. The rats were killed by decapitation about 48 hours after the last exposure. The brain and spinal cord (at the S5–S7 level) were rapidly removed and weighed. The brains were immediately dissected into cerebral hemisphere, cerebellum, and brainstem (medulla oblongata, pons, and peduncle) on an ice plate. Tissues of brain and spinal cord were kept frozen at –30°C until analysis of the marker proteins.

IMMUNOASSAY OF NERVE SPECIFIC MARKER PROTEINS

Tissues were homogenised with a teflon-glass homogeniser at 0°C in 10 volumes of 50 mM Tris HCl buffer (pH 7.5) containing 5 mM MgCl₂. The homogenates were centrifuged at 15 000 g for 20

minutes at 4°C. The supernatants were used for the assays of the nerve specific proteins, and for the estimation of soluble protein concentrations.

Two neuronal marker proteins (γ -enolase, calbindin-D28k) and three glial cell marker proteins (α -enolase, creatine kinase-B, β -S100 protein) were determined by means of sandwich type enzyme immunoassay systems. Details of these are described elsewhere.^{13–16} Briefly, they consisted of a solid phase (polystyrene balls) with immobilised rabbit antibodies monospecific to the respective subunit of enolase (γ - or α -enolase), creatine kinase-B, β -S100 protein, and calbindin-D28k, and the same antibodies labelled with β -D-galactosidase from *E. coli*.

The extract of the tissues was diluted 100 to 1000 fold with 0.01 M sodium phosphate buffer (pH 7.0, containing 0.1 M NaCl, 1 mM MgCl₂, 0.1% bovine serum albumin, and 0.1% NaN₃). Aliquots of 10 μ l of the diluted sample were subjected to immunoassay with the procedures described by Kato *et al.*¹⁰

Standard proteins used for the assays were purified rat $\gamma\gamma$ -enolase, $\alpha\alpha$ -enolase, creatine kinase-BB, calbindin-D28k, and human $\beta\beta$ -S100 protein. The values for each subunit of isoprotein are expressed as μ g or ng of the respective homodimeric isoprotein equivalent per mg soluble protein. Protein concentrations of the soluble fractions of homogenates were estimated by the dye binding method of Bradford¹⁷ using Bio-Rad reagents.

STATISTICAL ANALYSIS

The comparisons among multiple groups were performed using one way analysis of variance (ANOVA) followed by the Bonferroni method.¹⁸ A probability of $p < 0.05$ was accepted as significant.

Results

CLINICAL STATE, BRAIN WEIGHTS, AND TOTAL SOLUBLE PROTEIN CONCENTRATIONS

All animals survived the exposure period (16 weeks). Neither signs of peripheral neuropathy nor behavioural abnormalities were seen. We found no significant changes in body weight in any group of exposed animals compared with the controls. The

Table 1 Body and CNS weights of rats after chronic exposure to toluene

Regions	Control (mean (SE))	100 ppm (mean (SE))	300 ppm (mean (SE))	1000 ppm (mean (SE))
Body	508 (21)	503 (45)	493 (38)	489 (34)
Whole brain	2.14 (0.07)	2.15 (0.06)	2.14 (0.10)	2.12 (0.07)
Cerebrum	1.26 (0.06)	1.27 (0.05)	1.25 (0.07)	1.20 (0.04)
Cerebellum	0.29 (0.02)	0.29 (0.01)	0.29 (0.01)	0.29 (0.02)
Brainstem	0.57 (0.03)	0.57 (0.06)	0.58 (0.04)	0.59 (0.03)

No significant difference was found between exposed animals and controls.

For each group $n = 8$; all results are g.

weights of whole brain, cerebrum, cerebellum, and brainstem did not differ significantly among the four groups (table 1).

Total soluble proteins per wet weight in the cerebrum and cerebellum were not significantly different between exposed rats and controls. The concentrations of soluble protein in the brainstem and spinal cord were increased only in the 1000 ppm group (table 2).

Table 2 Concentrations of total soluble proteins in rat CNS after chronic exposure to toluene

Regions	Control (mean (SE))	100 ppm (mean (SE))	300 ppm (mean (SE))	1000 ppm (mean (SE))
Cerebrum	25.8 (0.9)	24.2 (0.8)	25.8 (1.6)	22.5 (0.6)
Cerebellum	24.9 (1.1)	22.7 (0.4)	22.8 (0.2)	22.8 (0.4)
Brainstem	17.9 (0.5)	19.1 (0.2)	19.7 (0.2)	21.9 (0.5)*
Spinal cord	9.3 (0.2)	10.2 (0.3)	10.4 (0.2)	12.3 (0.4)*

*p < 0.05; significantly different from control.
For each group n = 8; all results are mg/g wet tissue.

EFFECTS OF CHRONIC EXPOSURE TO TOLUENE ON NEURONAL MARKER PROTEINS (γ -ENOLASE, CALBINDIN-D28K)

The two neuronal markers did not show any significant changes in cerebrum and brainstem (table 3). In the cerebellum, γ -enolase concentration showed a dose dependent increase, whereas calbindin-D28k concentration remained unchanged. In the spinal cord, γ -enolase concentration was not significantly changed at any dose, whereas calbindin-D28k concentration was significantly decreased (17%) in the 1000 ppm group (p < 0.05).

EFFECTS OF CHRONIC EXPOSURE TO TOLUENE ON GLIAL CELL MARKER PROTEINS (α -ENOLASE, CREATINE KINASE-B, β -S100 PROTEIN)

An overall dose dependent increase in the amount of all three glial cell markers was seen in the cerebellum (table 4). A statistically significant increase was noted with exposure to 100 ppm toluene; a more pronounced increase occurred at the higher concentrations. Of the three markers examined, β -S100 protein

Table 3 Content of neuronal marker proteins in rat CNS after chronic exposure to toluene

	Control (mean (SE))	100 ppm (mean (SE))	300 ppm (mean (SE))	1000 ppm (mean (SE))
Cerebrum:				
γ -Enolase	10.0 (0.3)	9.8 (0.3)	9.7 (0.2)	10.6 (0.3)
Calbindin-D28k	540 (13)	537 (11)	565 (15)	555 (11)
Cerebellum:				
γ -Enolase	9.6 (0.4)	10.7 (0.3)*	11.0 (0.3)**	11.2 (0.3)**
Calbindin-D28k	4.15 (0.14)	4.45 (0.11)	4.56 (0.08)	4.55 (0.17)
Brainstem:				
γ -Enolase	11.7 (0.2)	11.6 (0.1)	11.2 (0.2)	10.9 (0.3)
Calbindin-D28k	383 (12)	376 (17)	359 (11)	363 (13)
Spinal cord:				
γ -Enolase	16.2 (0.7)	16.9 (0.5)	14.9 (0.9)	15.4 (0.5)
Calbindin-D28k	196 (3)	185 (8)	188 (4)	167 (8)*

*p < 0.05; **p < 0.01; significantly different from control.
For each group n = 8. Results for γ -enolase expressed as μ g/mg soluble protein; for calbindin-D28k as ng/mg soluble protein.

Table 4 Content of glial cell marker proteins in rat CNS after chronic exposure to toluene

	Control (mean (SE))	100 ppm (mean (SE))	300 ppm (mean (SE))	1000 ppm (mean (SE))
Cerebrum:				
α -Enolase	15.4 (0.4)	16.0 (0.7)	16.3 (0.4)	16.3 (0.5)
Creatine kinase-B	19.5 (0.6)	20.3 (0.5)	19.7 (1.0)	20.4 (0.6)
β -S100 Protein	3.29 (0.17)	3.60 (0.25)	4.02 (0.23)	3.67 (0.28)
Cerebellum:				
α -Enolase	14.7 (0.3)	16.7 (0.6)*	17.6 (0.5)**	17.7 (0.5)**
Creatine kinase-B	26.9 (0.7)	30.6 (0.8)**	32.0 (0.8)**	32.4 (0.8)**
β -S100 Protein	2.96 (0.12)	3.78 (0.32)	4.45 (0.12)**	4.79 (0.33)**
Brainstem:				
α -Enolase	18.8 (0.6)	20.7 (0.8)	19.7 (0.4)	19.6 (0.7)
Creatine kinase-B	23.5 (0.6)	25.8 (0.5)	23.2 (0.7)	22.0 (0.8)
β -S100 Protein	4.13 (0.22)	4.71 (0.26)	5.17 (0.28)*	5.40 (0.30)**
Spinal cord:				
α -Enolase	26.0 (1.0)	25.6 (1.2)	26.8 (0.8)	24.0 (1.6)
Creatine kinase-B	40.2 (1.1)	36.8 (1.7)	37.1 (1.0)	34.5 (0.8)*
β -S100 Protein	4.25 (0.24)	4.14 (0.17)	4.99 (0.32)	5.74 (0.25)**

*p < 0.05; **p < 0.01; significantly different from control.
For each group n = 8; all results are μ g/mg soluble protein.

showed the largest quantitative enhancement (28%, 50%, and 62% relative to control concentrations in the 100 ppm, 300 ppm, and 1000 ppm groups respectively). By contrast with the response pattern in the cerebellum, no significant changes in glial cell markers were found in the cerebrum. For the brainstem and spinal cord, only β -S100 protein concentration was significantly increased in a dose dependent manner. On the other hand, creatine kinase-B concentration showed a significant decrease (16%) in the spinal cord of the 1000 ppm toluene group.

Discussion

There is little doubt that prolonged exposure to high concentrations of toluene such as may occur during glue sniffing can damage the cerebellum and impair the higher integrative functions of the CNS. The effects of chronic low exposure, however, remain controversial, although some neurobehavioural studies suggest impaired functions of the CNS such as disturbances in learning ability, memory loss, changes in balance and coordination of locomotor movements, and high frequency hearing loss.¹ The biochemical basis for these effects and the dose-response relation have not been sufficiently explored. Limited pathological examinations have failed to show changes in brain structure after chronic inhalation of 1500–4000 ppm toluene.^{19,20} Therefore, the neurochemical approach becomes important to establish sensitive and specific indicators for the evaluation of this kind of solvent related CNS toxicity.

The present study provides biochemical evidence that long term inhalation of toluene can induce dose dependent changes in nerve specific marker proteins in the rat CNS. No reduction in the concentrations of total soluble protein was found (table 2); this excludes the possibility that quantitative enhancement of markers is due to diminished total protein content. Exposure to as low as 100 ppm toluene, which is the threshold limit value (TLV) set by the American Conference of Governmental Industrial Hygienists and the Japan Industrial Health Association, was found to produce a significant effect on glial cell markers in the cerebellum (table 4). Euler *et al*⁴ reported that exposure of old rats to toluene for three months at doses of 80 ppm could induce region specific effects on pre and postsynaptic parameters of catecholamine and neuropeptide transmission. Some epidemiological evidence also exists for an increased risk of neuropsychiatric disorders among workers chronically exposed to toluene below or around the TLV.^{21,22} The present study is in accordance with these investigations, which suggest that chronic low exposure to toluene around the TLV might have a potentiating effect on the CNS.

We also noted that the patterns of neurotoxic response to chronic exposure to toluene differ in

different areas of the CNS. The fact that the most pronounced increase in glial cell markers occurred in the cerebellum agrees with results of our previous subacute experiment.¹¹ Many case reports of toluene sniffing show that cerebellar abnormalities are the most commonly and convincingly described disorders among the persistent neurological deficits attributed to high exposure to toluene.²³ Some clinical investigations on industrial workers²⁴ and animals²⁵ also suggest the involvement of a cerebellar mechanism in toluene or toluene containing solvent induced disturbance of the vestibulo-oculomotor system. The present results support these clinical observations, and indicate the vulnerability of the cerebellum to toluene in animals under conditions comparable to occupational exposure. As well as the effects on the cerebellum, brainstem, and spinal cord, we also found dose dependent changes in β -S100 protein concentration. By contrast, amounts of marker substances examined in the cerebrum remained the same whatever the dose of toluene. This suggests that the cerebrum is less vulnerable to toluene than other areas of the CNS.

The enzyme immunoassay systems used are capable of identifying the specific cellular elements of neurones and glial cells. With the exception of calbindin-D28k in the spinal cord, the two neuronal cell markers did not show a quantitative decrease. This means that obvious neuronal loss or degeneration had not yet occurred by the time of death under the treatment schedule employed. Korbo *et al*²⁶ also reported that the total number of neurones in the cortex of rat brain did not show any decrease after 12 weeks of toluene administration. By contrast, the glial cell marker proteins displayed a definite dose dependent tendency to increase in the cerebellum, brainstem, and spinal cord. The development of gliosis is considered as an explanation for this phenomenon. Progressive reactions of the glial cells, including hypertrophy or proliferation, commonly follow damage to the CNS such as traumatic destruction, degenerative disorders, and infectious diseases,²⁷ and may result in the enhancement of the glial cell marker substances. The glial cell reactions after chronic exposure to chlorinated solvents, styrene, and toluene have also been proposed as an explanation by other authors.^{28–30} Gliosis may be a common and early finding indicative of solvent related effects on the CNS.

The possible mechanisms for gliosis include a primary action of neurotoxins on the glial cells, or a secondary indirect action responding to neuronal damage. The dose dependent increases in glial cell markers and the maintenance of neuronal markers in the rat CNS support the idea of direct action of toluene on glial cells.

It should be noted, however, that γ -enolase, a neurone-specific marker, also exhibited a significant

increasing trend in the cerebellum. Reactive astrocytes are reported to be capable of producing neurone-specific enolase that normally exists only in neurones.³¹ Therefore, the glial reaction induced by toluene treatment may partly contribute to the increase in γ -enolase concentration.

Interference with the energy metabolism of the CNS by toluene is speculated to be another explanation for the changes in γ -enolase, α -enolase, and creatine kinase-B concentrations, considering that all three proteins are glycolytic enzymes that play an important part in energy supply within the CNS. Sabri and Spencer³² indicated that disturbance of the energy metabolism may be one of the aetiological factors in the development of organic solvent neurotoxicity. The disruption of energy metabolism in the brain, which might occur under the high toluene exposure, and the heightened metabolic state arising from gliosis, may provoke the compensatory oversynthesis of glycolytic enzymes to satisfy the energy demand.

In summary, the present study found a dose dependent increase in glial cell markers and the relative maintenance of neuronal markers in the CNS of rats after chronic repeated exposure to toluene. This would mean that gliosis, rather than neuronal death, can be induced by toluene at exposure concentrations comparable with occupational conditions. Among the various CNS regions examined, the cerebellum is the most affected by treatment with toluene, whereas the cerebrum seems to be less vulnerable. Moreover, the significant changes induced by a dose as low as 100 ppm, together with the concentration dependent alterations, suggest that these nerve specific marker proteins may be used to evaluate solvent induced damage to the CNS.

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