Irreversible effects of dichloromethane on the brain after long term exposure: a quantitative study of DNA and the glial cell marker proteins S-100 and GFA

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ABSTRACT Two astroglial proteins S-100 and GFA, as well as DNA, were quantitatively determined in different regions of the gerbil brain after continuous long term exposure to moderate concentrations of dichloromethane. The intention of the experiment was to expose three groups of animals at three different solvent concentrations (210, 350, or 700 ppm) for three months. Because of the high mortality rate, however, the 700 ppm experiment was terminated after seven weeks. In the 350 ppm experiment half the exposed animals died and the exposure period was terminated after ten weeks. After the exposure period, the surviving gerbils in the 350 ppm exposure group and those from the 210 ppm group were allowed a postexposure solvent free period of four months. After exposure to 350 ppm, increased concentrations of the two astroglial proteins were found in the frontal and sensory motor cerebral cortex, compatible with astrogliosis in these regions. Exposure to 350 ppm and 210 ppm decreased the concentrations of DNA in the hippocampus. Moreover, after exposure at 350 ppm, DNA concentrations were also decreased in the cerebellar hemispheres. These results indicate a decreased cell density in these brain regions, probably due to cell loss. The neurotoxic effects were not found to correlate with the endogenous formation of carbon monoxide.

The adverse effects associated with exposure to dichloromethane (methylene chloride CH_2Cl_2) are primarily neurological and cardiovascular.^{1,2} Effects on the central nervous system are related to the anaesthetic properties of the solvent, and exposure of man to dichloromethane at 500-1000 ppm has been shown to cause early signs of central nervous system depression.^{1,3} Dizziness and lethargy, nausea, unconsciousness, and even death may result from accidental exposure to high concentrations.⁴

In the occupational setting diffuse toxic brain damage resulting in visual and auditory illusions has been reported after chronic exposure to dichloromethane.^{5,6} Bilateral temporal lobe degeneration has been described in one individual after long term occupational exposure to high concentrations of dichloromethane. The symptoms included loss of memory, mental deterioration, headache, and dysarthria; and an air encephalogram showed minor increases in subarachnoid air shadows in both frontal areas.⁷

Although several studies on animals have been performed after acute or short term exposure to dichlo-

romethane, to our knowledge none has been concerned with the biochemical or morphological effects on the central nervous system after chronic exposure.

The purpose of this study is to describe the effects of chronic dichloromethane exposure on the astroglial cell population in different brain regions by showing the altered concentrations of two astroglial cell marker proteins, S-100 and GFA.^{8,9} Traumatic damage to the central nervous system tissue is frequently a cause of hypertrophy or proliferation of astroglial cells, or both, as has been shown by immunohistochemical methods using specific antibodies to astroglial proteins.¹⁰⁻¹² Furthermore, astrogliosis as a consequence of trauma to the brain is well known in human neuropathology. Previous studies in our laboratory have shown that chronic exposure to trichloroethylene or ethanol can induce increased concentrations of S-100 in different regions of the gerbil brain compatible with the presence of astrogliosis.^{13,14}

The intention of the experiment was to expose gerbils by continuous inhalation to dichloromethane at 210, 350, or 700 ppm for three months, followed by a postexposure solvent free period of four months. After this last period, concentrations of S-100, GFA,

DNA, and protein in various regions of the brain were quantitatively measured.

A major toxicological hazard of dichloromethane in the brain during acute exposure is considered to be the formation of carbon monoxide during biotransformation.^{1,15} In a complementary study the carboxyhaemoglobin per cent saturation was determined in the blood of gerbils continuously exposed for three weeks at 210 and 350 ppm.

Materials and methods

MATERIALS

Barbital, sodium barbital, sodium azide, sodium chloride, Perhydrol (30% H₂O₂), tris, glycine, EDTA, and 2-mercaptoethanol were of analytical grade (Merck, Germany). Agarose HSA (Litex, Denmark), 96 well flat bottom microtest plates (NUNC, Denmark), peroxidase conjugated swine antirabbit IgG (Dako, Denmark), salmon DNA, bovine albumin, o-phenylenediamine, and diaminobenzidine (Sigma Chemical Co, USA) and nitrocellulose membranes, acrylamide, bisacrylamide, Commassie brilliant blue, and SDS (Bio-Rad, USA) were used. Commercial dichloromethane, cleaning grade, containing 0.3–0.5% stabilisers (butylene oxide and antioxidants) was obtained from Billerud AB (Sweden).

ANIMALS

Seventy six male and 60 female Mongolian gerbils (*Meriones unguiculatus*) were used. The initial mean weight of the male gerbils was 72.8 ± 1.9 (SE) and of the females 57.8 ± 1.0 (SE). Male and female animals were housed separately in transparent plastic cages (45 × 24 × 15 cm, four or five to a case). Experimental and control animals were sex matched littermates. Commercial laboratory rat food (Astra/Ewos, Sweden) and water were freely available. Sawdust was used as bedding. The environmental temperature was kept to $22^\circ\text{C} \pm 2^\circ\text{C}$. Light supplementing daylight was automatically controlled for 12 hours daylight, with half an hours twilight at dawn and evening.

EXPOSURE

During the experimental runs, exposure was continuous. Dichloromethane was injected into a temperature controlled glass vapouriser (60°C), mixed with a small volume of air (20 l/h), and subsequently diluted with clean air (1000 l/h) to produce the desired concentration. The concentration in the inhalation chambers was monitored with a Miran 1A spectrophotometer fitted with a multipath gas cell. All air was filtered to remove oil and particles larger than 0.3 µm. The long term stability of the system permitted a concentration of solvent to be held within 10% of the

target concentration. Interruptions occurred for only one to two hours a week for changing of water, bedding material, and food. Control animals were exposed to clean air (1000 l/h).

Three experiments were performed: gerbils were exposed to 210, 350, or 700 ppm. At each solvent concentration 10 male and 10 female gerbils were exposed; 60 sex matched littermates served as controls. The intention was to expose the experimental groups for three months. Because, however, of the high mortality rate in the 700 ppm exposure group, this exposure was terminated after seven weeks. In the 350 ppm group half the animals died during the ninth and tenth week and as a result the exposure of this group was terminated after 10 weeks. The 210 ppm group was exposed as planned for three months. The littermate controls were housed in chambers identical to those of the experimental animals throughout the experimental period. After the exposure period, all surviving animals were removed from the inhalation chambers and kept free of exposure for four months.

PREPARATION OF TISSUE

After the solvent free period, four male and four female gerbils from each of the two remaining experiments and 16 littermate controls, were killed. The brains were removed and dissected on ice. The cerebral cortices were divided lengthwise into three equal parts, frontal, middle, and dorsal. The cerebellar hemispheres were divided at the position of the sulci intercrurales into anterior and posterior parts. The anterior (lobuli I–V) and posterior (lobuli VI–X) cerebellar vermis and the brainstem (under the 4th ventricle) and the hippocampus were also dissected. Tissue samples were weighed, then quickly frozen on solid ice and stored in tight fitting boxes at -80°C until analysed. Tissues were homogenised at 1:5 or 1:20 (wt/vol) in 0.024 M barbital buffer, pH 8.6, with 2.5 mM EDTA and 1.0 mM 2-mercaptoethanol; they were then centrifuged at 50 000 g for 60 minutes.

ANALYTICAL PROCEDURE

Protein concentrations in the homogenates were determined according to Lowry *et al.*¹⁶ Bovine serum albumin was used as a reference standard.

The S-100 concentrations were measured in the supernatants by rocket immunoelectrophoresis.¹⁷ Bovine S-100 was used as a reference standard.

Analyses of the DNA concentrations in the homogenates were carried out according to Kissane and Robins.¹⁸ Salmon DNA was used as a reference standard.

GFA concentrations were determined in the supernatants by a solid phase ELISA-technique.¹⁹ A first layer, normal gerbil brain supernatant in incubation buffer (0.024 M barbital buffer, pH 8.6, containing

1.0 mM 2-mercaptoethanol, 2.5 mM EDTA, 1.0 U/ml aprotinin, and 0.1% sodium azide) was absorbed on to flat bottom polystyrene microtest plates by incubating overnight at +4°C. A total of 5.4 µg brain protein was applied per well. The plates were washed in 0.15 M NaCl, containing 0.05% (v/v) TWEEN-20 and incubated with 0.5% albumin (w/v) in incubation buffer for four hours at room temperature, and they were subsequently washed again. After 50 µl incubation buffer had been added to each well, followed by 5 µl samples or reference gerbil supernatant, 50 µl anti-GFA diluted 1:4000 was subsequently added to each well and the plates were incubated for five hours at room temperature. After washing, peroxidase conjugated swine antirabbit IgG, diluted 1:200, was added to each well and allowed to incubate overnight at +4°C. The plates were then washed and enzyme substrate was added: o-phenylenediamine, 0.5 mg/ml in 50 mM citrate buffer, pH 5.0, containing 0.01% H₂O₂. The reaction was stopped with 1.0 M H₂SO₄ and the absorbance was measured at 490 nm in an automatic ELISA-reader (Flow, USA). The arbitrary unit (AU) used refers to the amount of GFA in 1 µl of the reference gerbil supernatant. Experiments in our laboratory indicate that the extraction procedure used allows solubilisation of about a quarter of the total GFA in gerbil brain tissue (unpublished observation), which is in good agreement with the findings of Gheuens *et al.*²⁰

ANTIBODIES

The S-100 antibodies were prepared according to Haglid *et al.*²¹ The GFA antibodies were prepared according to Dahl and Bignami.²² The specificity of the antibodies to GFA was demonstrated by immunoblotting. Samples of the reference gerbil supernatant containing 54 µg, 162 µg, and 270 µg protein were run in SDS gel electrophoresis, performed in a 5–10% linear gradient polyacrylamide slab gel.²³ The proteins were transferred to a nitrocellulose membrane according to Towbin *et al.*²⁴ except that 0.1% SDS was added to the transfer-buffer. The electrophoretic blots were detected using anti-GFA (diluted 1:10000 in tris buffered saline) as the first antibody and peroxidase conjugated swine antirabbit IgG (diluted 1:200) as the second antibody. Diaminobenzidine (0.5 mg/ml in tris buffered saline) and H₂O₂ (0.03%) was used as enzyme substrate for the colour reaction.

DETERMINATIONS OF CARBOXYHAEMOGLOBIN PER CENT SATURATION

Eight littermate male pairs of gerbils were continuously exposed to dichloromethane for three weeks; eight were exposed at 210 ppm and the lit-

termates at 350 ppm. At the end of the exposure the animals were removed from the inhalation chambers and immediately bled. Carboxyhaemoglobin per cent saturation was determined according to Collison *et al.*²⁵ as modified by Lundh *et al.*²⁶

STATISTICAL EVALUATION

Fisher's non-parametric permutation test for paired observations was used.²⁷

Results

MORTALITY, BODY AND BRAIN WEIGHTS

The exposure at 700 ppm was terminated after seven weeks because of the high mortality rate among the exposed animals (fig 1). Between day 28 and day 52 of the experiment, 10 male and nine female gerbils died. The mean survival time in days of these animals was 38.6 ± 2.0 (SE) for male and 36.1 ± 3.3 (SE) for female animals. No control animals died during the experimental period. During the exposure period the exposed animals lost weight compared with the controls (fig 1).

The exposure at 350 ppm was terminated after 10 weeks, also because of the high mortality rate among the exposed animals (fig 1). Between days 63 and 71 of the experiment six male and three female gerbils died.

Fig 1 Weight development among dichloromethane exposed gerbils at three different exposure levels. ♂: male gerbils. ♀: female gerbils. —: weight. ---: ppm. +: indicates death of one gerbil.

The mean survival time in days of these animals was 67.5 ± 1.5 (SE) for male and 66.0 ± 1.5 (SE) for female animals. No control animals died during the experiment. A depressed weight gain was observed among the exposed animals during exposure (fig 1). At the end of the exposure period (day 70) the exposed animals weighed significantly less compared with the littermate controls ($p \leq 0.05$). During the postexposure period, however, the exposed animals gained weight and at the end of the experiment exposed and control animals had similar weights (fig 1).

The exposure at 210 ppm was continued for three months as planned. One exposed female gerbil died at day 88. No control animals died. During the exposure period, the exposed animals gained weight normally and no significant difference in weight between exposed and control animals was observed at the end of the exposure period or at the end of the experiment.

Brain weights did not differ between control and exposed animals, neither after exposure at 210 ppm nor after exposure at 350 ppm. The weights of the dissected brain areas were significantly increased in the cerebral sensory motor cortex after exposure at 350 ppm ($p \leq 0.05$). The weights of the other brain areas at this exposure level and 210 ppm were not significantly changed ($p > 0.05$).

S-100 PROTEIN

The S-100 concentrations per wet weight in the different brain areas studied were not significantly altered after exposure to dichloromethane at 210 ppm. Increased concentrations were found in the cerebral frontal cortex and in the sensory motor cortex after exposure at 350 ppm, however (fig 2).

GFA PROTEIN

The GFA concentrations per wet weight were significantly raised in both the cerebral frontal cortex and the cerebral sensory motor cortex in the animals exposed to dichloromethane at 350 ppm. The GFA concentrations per wet weight, however, were not significantly altered in these areas after exposure at 210 ppm (fig 3).

DEOXYRIBONUCLEIC ACID

DNA concentrations per wet weight were significantly decreased in the hippocampus after exposure at 210 and 350 ppm dichloromethane. The DNA concentrations were also found to be decreased in the cerebellar hemispheres after exposure to 350 ppm (fig 4).

PROTEINS

Protein concentrations per wet weight in the different

Fig 2 S-100 protein concentrations per wet weight (ng/mg) in gerbil brain areas after exposure to dichloromethane at (a) 210 ppm for three months and (b) 350 ppm for two months, followed by a postexposure solvent free period of four months. F: frontal cerebral cortex; SM: sensory motor cerebral cortex; O: occipital cerebral cortex; HA: anterior cerebellar hemispheres; HP: posterior cerebellar hemispheres; VA: anterior cerebellar vermis; VP: posterior cerebellar vermis; BS: brain stem; HIP: hippocampus. Bars represent mean values of eight animals and SEM. * $p < 0.05$; ** $p < 0.01$.

regions studied were not significantly changed after exposure to dichloromethane when compared with controls.

To exclude the possibility that the changes observed regarding S-100, GFA, and DNA concentrations per wet weight are due to a changed dry/wet weight ratio in the tissue samples, S-100, GFA, and DNA were also calculated as concentrations per protein. These results (fig 5) are in agreement with the results as calculated per wet weight (figs 2–4).

IMMUNOBLOTTING OF REFERENCE SUPERNATANT

Only one band was found to be GFA immunoreactive when the reference gerbil brain supernatant was subjected to SDS-electrophoresis and transferred to a nitrocellulose membrane (fig 6).

Fig 3 GFA protein concentrations per wet weight (AU/mg) in gerbil brain areas after exposure to dichloromethane at (a) 210 ppm for three months and (b) 350 ppm for two months, followed by a postexposure solvent free period of four months. F: frontal cerebral cortex; SM: sensory motor cerebral cortex; O: occipital cerebral cortex; HA: anterior cerebellar hemispheres; HP: posterior cerebellar hemispheres; VA: anterior cerebellar vermis; VP: posterior cerebellar vermis; BS: brain stem; HIP: hippocampus. Bars represent mean values of eight animals and SEM. * $p < 0.05$; ** $p < 0.01$.

CARBOXYHAEMOGLOBIN PER CENT SATURATION

No significant difference in carboxyhaemoglobin saturation was observed among the animals continuously exposed to dichloromethane for three weeks. The mean value for the 210 ppm group was 11.5 ± 0.4 (SE) and for the 350 ppm group 10.3 ± 0.7 (SE).

Discussion

In this study the levels of exposure were three, five, and ten times the current Swedish threshold limit value (TWA: 70 ppm). The intention of the experiments was to expose the animals by continuous inhalation for three months, as previous studies have shown this period to be sufficient to induce lasting alterations in different brain regions after exposure to trichloroethylene and ethanol.^{13 14} Because of the 95% mortality rate in the 700 ppm group during the second month of exposure, however, this experiment was terminated after seven weeks of exposure, and as the mortality rate in the 350 ppm group was 45% at the beginning of the third month, the exposure was terminated after 10 weeks. Only the 210 ppm group was exposed for three months as planned. As in our previous studies of the chronic neurotoxicity of trichloroethylene and ethanol,^{13 14} the surviving animals were then subjected to a solvent free post-exposure period of four months to allow sufficient time for regeneration and restoration of neuronal function.²⁸ The high mortality among the gerbils had not been expected and no necropsy of these animals

Fig 4 DNA concentrations per wet weight (μ g/mg) in gerbil brain areas after exposure to dichloromethane at (a) 210 ppm for three months and (b) 350 ppm for two months, followed by a postexposure solvent free period of four months. F: frontal cerebral cortex; SM: sensory motor cerebral cortex; O: occipital cerebral cortex; HA: anterior cerebellar hemispheres; HP: posterior cerebellar hemispheres; VA: anterior cerebellar vermis; VP: posterior cerebellar vermis; BS: brain stem; HIP: hippocampus. Bars represent mean values of eight animals and SEM. * $p < 0.05$.

Fig 5 (a) S-100 concentrations per protein (µg/mg), (b) GFA concentrations per protein (AU/mg), and (c) DNA concentrations per protein (µg/mg) in gerbil brain areas after exposure to dichloromethane at 350 ppm for two months, followed by a postexposure solvent free period of four months. F: frontal cerebral cortex; SM: sensory motor cerebral cortex; O: occipital cerebral cortex; HA: anterior cerebellar hemispheres; HP: posterior cerebellar hemispheres; VA: anterior cerebellar vermis; VP: posterior cerebellar vermis; BS: brain stem; HIP: hippocampus. Bars represent mean values of eight animals and SEM. *p < 0.05; **p < 0.01.

was performed. The relatively few long term exposure studies using dichloromethane reported have not described this phenomenon²⁹; on the contrary, the solvent has been considered relatively harmless. To

our knowledge, however, none of these studies has used continuous exposure but has been workday related. Moreover, as dichloromethane has a high vapour pressure and is relatively water soluble, but

Fig 6 Electrophoretic separation in a SDS polyacrylamid gradient gel (5–10%). Lane (a): molecular weight standards (from above: phosphorylase b: 94 000, bovine serum albumin: 67 000, ovalbumin: 43 000, carbonic anhydrase: 30 000, soyabean trypsin inhibitor: 20 100, α-lactalbumin: 14 400). Lane (b): the reference gerbil supernatant containing 27 µg protein. Stained with Commassie brilliant blue. Electrophoretograms of samples of the reference gerbil supernatant containing (c): 54 µg, (d): 162 µg, and (e): 270 µg protein; subjected to electrophoresis as in (b). Detection was performed using anti-GFA (diluted 1:10 000) as first antibody and peroxidase conjugated swine antirabbit IgG as second antibody. Diaminobenzidine was used for colour reaction.

less lipid soluble than other chlorinated solvents,³⁰ it is rapidly exhaled after exposure. Thus in workday related long term exposure experiments there should be only a slight risk of accumulation of dichloromethane in the lipid rich tissues of the experimental animal. During continuous exposure experiments, however, accumulation of dichloromethane in the animal tissues is probable, especially if a limited metabolising capacity of the solvent is postulated.³¹ These findings necessitate further studies comparing the adverse effects of the solvent on the living organism after continuous and workday related exposure experiments.

In man certain brain areas seem to be more vulnerable to chronic dichloromethane exposure.⁷ In previous studies a varying sensitivity of different brain regions to changes in glial cell S-100 protein has been shown after exposure to trichloroethylene^{13 21} and ethanol.¹⁴ In order to elucidate this discrete brain regions were investigated in this study.

The S-100 protein is mainly found in protoplasmic and fibrillary astrocytes of the brain.³² Hence, the localisation and the high solubility of the S-100⁹ makes this protein a suitable marker for an astroglial cell increase in response to brain damage.^{12 33 34} The GFA protein is the main component of the astroglial filaments.^{8 35} Thus GFA is a suitable marker for an astroglial fibre increase in response to brain injury.^{10 11 36}

Consequently, the increased concentrations of S-100 and GFA observed in the frontal cerebral cortex and the sensory motor cerebral cortex, after the 350 ppm exposure, are compatible with an astroglial hypertrophy or proliferation, or both, and with an astrogliosis in these regions. Hypothetically, the increased concentrations of GFA could also reflect a redistribution between soluble and insoluble GFA, due either to disassembly³⁷ or to proteolytic degradation of the glial filaments.³⁸ As the concentrations of S-100 and GFA are increased simultaneously, however, this seems unlikely.

Assuming a diploid DNA content in the brain cells, and a metabolic stability of DNA in an adult brain,³⁹⁻⁴¹ the DNA concentration represents a measure of the mean density of cells, reflecting the different cellular components of the central nervous system. In this context a concomitant increase of DNA and astroglial protein concentrations could possibly be used as an indication of astroglial proliferation in response to brain injury. In this study, however, the DNA concentrations were not increased in the frontal cerebral cortex and the sensory motor cerebral cortex; on the contrary, decreased DNA concentrations were obvious in the hippocampus after exposure to both 210 and 350 ppm and also in the cerebellar hemispheres after the 350 ppm exposure.

These observations indicate a decreased cell density in these brain regions that may be due to a loss of cells. Cell loss might be ascribed to cell death of, for example, nerve or oligodendroglial cells, or inhibition of the slow non-neuronal cell acquisition phase in the adult brain.⁴² Inhibition of DNA synthesis by dichloromethane has previously been described in a tissue culture system, using hamster epithelial cells and human fibroblasts.⁴³ Cellular events occurring during changes of the normal environment of brain cells, especially with regard to neurotoxins, is obviously complex. In areas with increased astroglial protein concentrations, a proliferation of astroglial cells might contribute to "normalised" DNA concentrations, whereas a loss among other cells than astroglia could mask a rise in DNA concentrations. Accordingly, after exposure at 350 ppm, no brain area simultaneously displayed increased concentrations of astroglial proteins and decreased concentrations of DNA.

It is not known whether the astroglial reactivity observed is caused by a primary action of dichloromethane on the astroglial cells, or is an indirect reaction in response to, for example, neuronal damage. Astroglial proteins have previously been used to show the occurrence of progressive astroglial reactivity both as a consequence of nerve cell injury in retrograde degeneration of the thalamic nuclei,³³ and in response to direct tissue damage such as cortical stab wounds.^{10 11}

The mechanism behind the neurotoxicity of dichloromethane is unclear. Since dichloromethane is lipid soluble,³⁰ interference with the biological membranes of the brain could be one cause of the biochemical alterations observed. Furthermore, the relation between the metabolism of dichloromethane and the neurotoxicity of the compound is not fully understood. Dichloromethane is metabolised in the rat liver by two major pathways of biotransformation. The first pathway described follows oxidation by the microsomal cytochrome P-450 mono-oxygenases, ultimately yielding carbon monoxide⁴⁴; the second follows conjugation to glutathione leading to formation of formaldehyde.⁴⁵ In a complementary experiment carboxyhaemoglobin concentrations were determined in gerbils exposed to 210 or 350 ppm dichloromethane by continuous inhalation. Assuming that three weeks exposure renders steady state carboxyhaemoglobin concentrations, the similar carboxyhaemoglobin concentrations observed at these two exposure levels indicate a limited metabolising capacity of the microsomal cytochrome P-450 mono-oxygenases, in agreement with earlier studies.^{29 31} As the biochemical alterations induced in the gerbil brain were only minor after exposure to 210 ppm, but pronounced after exposure to 350 ppm,

these results do not favour the hypothesis that carboxyhaemoglobin induced cerebral hypoxia is the major cause of dichloromethane induced brain dysfunction as previously suggested.⁷

As both the major pathways of dichloromethane metabolism are present to some extent in the rat brain,^{45,46} local metabolite formation could be of importance to explain the neurotoxicity of the compound. Dichloromethane derived ¹⁴C activity has been described in the rat brain 48 hours after a short time exposure, probably reflecting binding of metabolites to macromolecules or to the incorporation of dichloromethane derived one carbon fragments into normal metabolic pathways.^{31,47} One metabolite, formaldehyde, is a well recognised toxin, which is known to bind covalently to tissue macromolecules.⁴⁸

It is suggested that astroglial reactions are a common feature after long term exposure to some solvents,^{13,14,49} as well as to dichloromethane, and that the changes observed are irreversible as they occur despite a postexposure solvent free period. These alterations serve as an indicator of solvent induced brain damage and they are in accordance with the signs of diffuse toxic brain damage described by von Hanke *et al*⁵ and von Weiss⁶ after chronic occupational exposure to dichloromethane.

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