Scand J Work Environ Health 11 (1985) 447—455

Astrogliosis in the cerebral cortex of gerbils after long-term exposure to 1,1,1-trichloroethane

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ROSENGREN LE, AURELL A, KJELLSTRAND P, HAGLID KG. Astrogliosis in the cerebral cortex of gerbils after long-term exposure to 1,1,1-trichloroethane. *Scand J Work Environ Health* 11 (1985) 447—455. Mongolian gerbils *(Meriones ungiculatus)* were continuously exposed by inhalation to 1,1,1-trichloroethane at 70, 210, or 1 000 ppm for three months, followed by a four-month postexposure solvent-free period. Concentrations of two astroglial proteins, S-100 and glial fibrillary acidic (GFA) protein, were then determined in different regions of the cerebral cortex. The main biochemical alterations induced after exposure to 210 and 1 000 ppm of 1,1,1-trichloroethane demonstrated a pronounced change in gerbil brain; increased concentrations of GFA protein were found in the cerebral sensorimotor cortex at both these exposure levels, an occurrence indicating astrogliosis in this brain region. These results suggest that 1,1,1-trichloroethane should not be regarded as harmless, particularly regarding neurotoxicity, as previously claimed.

Key terms: chronic exposure, glial fibrillary acidic protein, neurotoxicity, S-100.

1,1,1-Trichloroethane (methylchloroform) is a chlorinated hydrocarbon that is widely used in industrial and consumer products. It has become increasingly popular during the last few decades because of its reputed low degree of toxicity (49). However, little is known of the neurotoxicity of the compound. Studies on workers in occupational settings have been made, and no adverse effects on the central nervous system were observed after chronic exposure (24, 35). However, the exposure levels in these studies were relatively low. A short-term exposure of humans to 1,1,1-trichloroethane at 350 ppm has been shown to impair functions of the central nervous system in some individuals (18). Lightheadedness, headache, problems with coordination, drowsiness, anesthesia, narcosis, and even death may occur as a consequence of accidental human exposure to high concentrations of the solvent (12, 36, 50, 51).

A few long-term studies on animals have been performed, and 1,1,1-trichloroethane has been reported to be tolerated in several different species after long-term exposures at different solvent concentrations (14, 41). However, to our knowledge, no long-term studies concerning the effects on the central nervous system exist.

The central nervous system is known to be a target organ for other organic solvents during long-term exposure. Previously, trichloroethylene (21) and ethanol (42) have been shown to induce irreversible effects in the astroglial cell population of the brain. The purpose of this study was to describe the effects of chronic 1,1,1-trichloroethane exposure on the astroglial cell population in the gerbil cerebral cortex. Gerbils were exposed by continuous inhalation to 1,1,1-trichloroethane at 70, 210, and 1 000 ppm for three months. This period was followed by a postexposure solvent-free period of four months. Thereafter, concentrations of the astroglial proteins S-100 (39) and glial fibrillary acidic (GFA) protein (5, 15) were quantitatively analyzed in different parts of the cerebral cortex.

Materials and methods

Materials

Barbital, sodium barbital, sodium azide, sodium chloride, Perhydrol [30 % hydrogen peroxide (H_2O_2)], tris(hydroxymethyl)aminomethane (tris), glycine, ethylenediaminetetraacetate (EDTA), and 2-mercaptoethanol were of analytical grade (Merck, Germany). Agarose (HSA, Litex, Denmark), microtest plates with 96 flat-bottom wells (NUNC, Denmark), swine anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Dako, Denmark), bovine albumin, o-phenylenediamine, and diaminobenzidine (Sigma Chemical Co, United States), as well as nitrocellulose membranes, acrylamide, bisacrylamide, Coomassie brilliant blue, and sodium dodecyl sulfate (SDS) (Bio-Rad, United States), were used. Commercial 1,1,1-trichloroethane (cleaning grade), containing 5 % dioxane-free stabilizers (methyl ethyl ketone, methylene oxide, butylene epoxide, butanol and nitromethane), was obtained from Billerud AB (Sweden).

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Animals

Twenty-four male and 24 female Mongolian gerbils *(Meriones ungiculatus)* were used. The initial mean weight of the male gerbils was 65.4 (SE 1.0) g and of the female 53.0 (SE 0.9) g. Male and female animals were housed separately in transparent plastic cages (45 × 24 × 15 cm, four in each). The experimental and control animals were sex-matched littermates. Commercial laboratory rat chow (Astra/Ewos, Sweden) and water was freely available. Sawdust was used as bedding. The environmental temperature was kept at 22 ± 2°C. Light supplementing daylight was automatically controlled for 12 h of daylight, with 30 min of twilight at dawn and evening.

Exposure

During the experimental runs, the exposure was continuous. 1,1,1-Trichloroethane was injected into a temperature-controlled glass-vaporizer (60°C), mixed with a small volume of air (20 l/h), and then diluted with clean air (1 000 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 the concentration of the solvent to be held within 10 % of the decided concentration. Interruptions occurred only 1—2 h per week for the changing of water, bedding material, and food.

Three experiments were performed; gerbils were exposed to a concentration of 70, 210, or 1 000 ppm. The exposure periods were for three months. At each solvent concentration four male and four female gerbils were exposed. The 24 sex-matched littermates served as controls. The littermate controls were housed in chambers identical to those of the experimental animals throughout the experimental period. After the exposure period, all the animals were removed from the inhalation chambers and kept free from exposure for a four-month period.

Preparation of tissue

After the solvent-free period, the gerbils were randomly selected to be decapitated. The brains were removed from the skulls and separated from the spinal cords by transection at the distal end of the fourth ventricle. The olfactory bulbs were removed, and the brains were weighed. Cerebral cortices were dissected on ice and divided by transverse cuts into three equally long parts, ie, frontal (frontal cerebral cortex), middle (sensorimotor cerebral cortex), and dorsal (occipital cerebral cortex) parts. The tissue samples were weighed, quickly frozen on dry ice, and then stored in tight-fitting boxes at —80°C until analyzed. Tissues were homogenized at 1:5 (weight:volume) 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 min. The dissections and weighings were always carried out by the same persons, as were the biochemical analyses.

Analytical procedure

Protein concentrations in the homogenates were determined according to Lowry et al (30). Bovine serum albumin was used as the reference standard.

The S-100 concentrations were measured in the supernatants by rocket immunoelectrophoresis (48). Bovine S-100 was used as the reference standard.

The GFA protein concentrations were determined in the supernatants by a solid-phase enzyme-linked immunosorbent assay (ELISA) (16). 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 onto the microtest plates by incubation overnight at 4°C. A total of 5.4 μg of brain protein was applied per well. The plates were then washed in 0.15-M sodium chloride, containing 0.05 % (volume: volume) TWEEN-20 (polyoxyethylenesorbitan), and incubated with 0.5 % albumin (weight:volume) in incubation buffer for 1 h at room temperature, and then they were subsequently washed again. Incubation buffer (50 μl) was added to each well, followed by 5-μl samples or reference gerbil supernatant. Fifty microliters of anti-GFA diluted 1:4 000 was subsequently added to each well, and the plates were incubated 5 h 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 the enzyme-substrate (o-phenylenediamine, 0.5 mg/ml, in 50-mM citrate buffer, pH 5.0, containing 0.01 % H_2O_2) was added. The reaction was stopped with 1.0-M sulfuric acid, and the absorbance was measured at 490 nm in an automatic ELISA reader (MR 600, Dynatech, United States). The arbitrary unit (AU) used refers to the amount of GFA protein in 1 μl of the reference gerbil supernatant.

Antibodies

The S-100 antibodies were prepared according to Haglid et al (22). The GFA protein antibodies were prepared according to Dahl et al (9).

Immunoblotting

Samples were run in SDS gel electrophoresis, performed in a 6—12 % linear gradient polyacrylamide slab gel (27). The samples consisted of supernatants from the sensorimotor cerebral cortex of four gerbils exposed at 210 ppm, and the four corresponding controls, each calculated to contain 5 AU of GFA protein. The proteins were transferred to a nitrocellulose membrane according to Towbin et al (52), except that 0.1 % SDS was added to the transfer buffer. Electro-

phoretic blots were detected with the use of anti-GFA protein (diluted 1 : 10 000 in tris-buffered saline) as the first antibody and the swine antirabbit IgG conjugated with peroxidase (diluted 1 : 200) as the second antibody. Diaminobenzidine (0.5 mg/ml in tris-buffered saline) and H_2O_2 (0.03 %) was used as the enzyme-substrate for the color reaction.

Statistical evaluation

The nonparametric Fisher's permutation test for paired observations was used to test the differences observed between the control and exposed groups (6).

The differences in the concentrations of S-100 between the control and exposed animals for all brain regions, as well as the corresponding differences in the concentrations of GFA protein, were weighted together according to Mantel (33). The correlations between these differences in the S-100 and GFA protein concentrations were then tested with Fisher's permutation test.

Results

Body and brain weights

No animals died during the exposure or the solvent-free periods. There were no significant differences in body weight between the exposed animals and their respective controls, either at the end of the exposure or after the solvent-free period. The brain weights did not differ between the control and exposed animals after exposure at 70 and 210 ppm. However, after exposure at 1 000 ppm, the brain weights of the exposed animals were decreased when compared to those of the controls (table 1). The weights of the dissected brain regions were not significantly changed between the exposed and the control animals ($p > 0.05$). The standard errors of these weights were less than 5 % of the mean.

Concentrations of glial fibrillary acidic protein

The GFA protein concentrations per wet weight in the different brain regions studied were not significantly changed after exposure to 1,1,1-trichloroethane at 70 ppm. Increased concentrations of GFA protein were found in the sensorimotor cerebral cortex after exposure to both 210 and 1 000 ppm (figure 1).

Table 1. Brain weights of gerbils exposed to 1,1,1-trichloroethane and their respective controls — Mean of eight animals and the standard error (SE) of the mean.

Exposure level	Brain weights (g)			
	Exposed animals		Control animals	
	Mean	SE	Mean	SE
70 ppm	0.995	0.009	1.001	0.006
210 ppm	0.990	0.009	0.997	0.006
1 000 ppm	0.978	0.011	1.004	0.014**

** $p \leq 0.01$, Fisher's permutation test for paired observations.

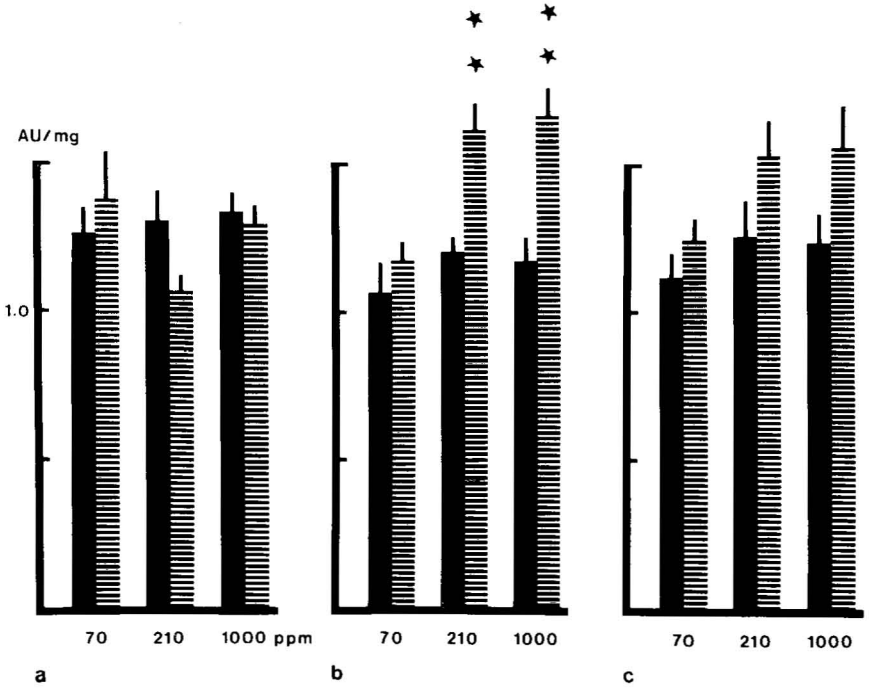

Figure 1. Concentrations of glial fibrillary acidic (GFA) protein per wet weight in three gerbil brain regions [frontal cerebral cortex (a), sensorimotor cerebral cortex (b), occipital cerebral cortex (c)] after exposure to 1,1,1-trichloroethane at 70, 210, and 1 000 ppm for three months followed by a four-month solvent-free period. The statistical evaluation was performed with Fischer's permutation test (** $p \leq 0.01$). [Black columns = values for control animals, striped columns = values for exposed animals, AU = arbitrary units (1 AU = amount of GFA protein in 1 μl of the reference gerbil supernatant)]

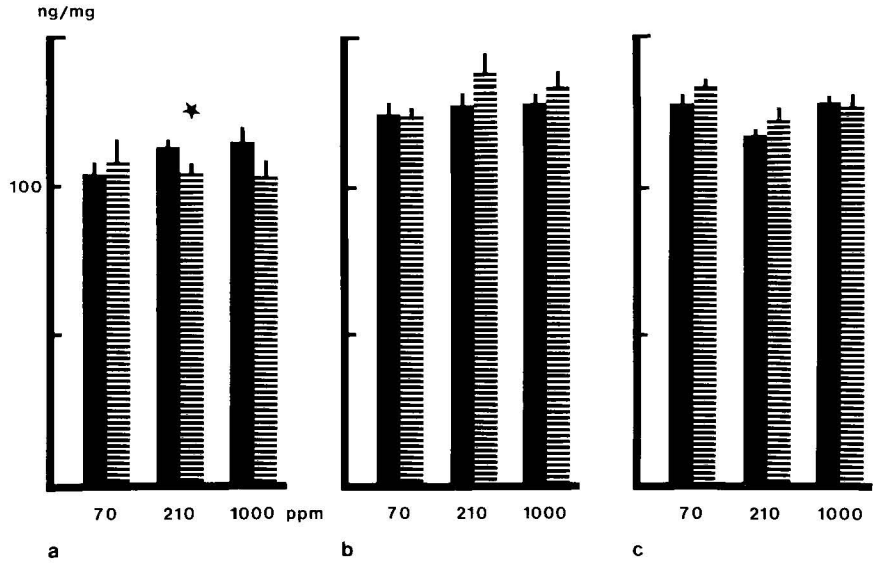

Figure 2. S-100 concentrations per wet weight in three gerbil brain regions [frontal cerebral cortex (a), sensorimotor cerebral cortex (b), occipital cerebral cortex (c)] after exposure to 1,1,1-trichloroethane at 70, 210, and 1 000 ppm for three months followed by a four-month solvent-free period. The statistical evaluation was performed with Fisher's permutation test (* $p \leq 0.05$). (Black columns = values for control animals, striped columns = values for exposed animals)

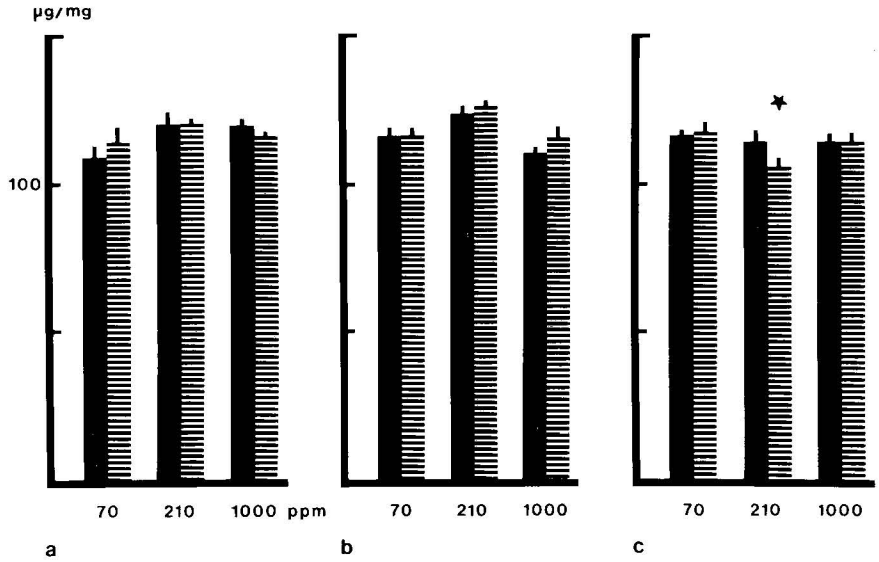

Figure 3. Protein concentrations per wet weight in three gerbil brain regions [frontal cerebral cortex (a), sensorimotor cerebral cortex (b), occipital cerebral cortex (c)] after exposure to 1,1,1-trichloroethane at 70, 210, and 1 000 ppm for three months followed by a four-month solvent-free period. The statistical evaluation was performed with Fischer's permutation test (* $p \leq 0.05$). (Black columns = values for control animals, striped columns = values for exposed animals)

S-100 concentrations

The S-100 concentrations per wet weight in the different brain regions studied were not significantly changed after exposure to 1,1,1-trichloroethane at 70 or 1 000 ppm. After exposure at 210 ppm, decreased concentrations of S-100 were found in the frontal cerebral cortex (figure 2).

Protein concentrations

Protein concentrations per wet weight in the different brain regions studied were not significantly changed after exposure to 1,1,1-trichloroethane at 70 and 1 000

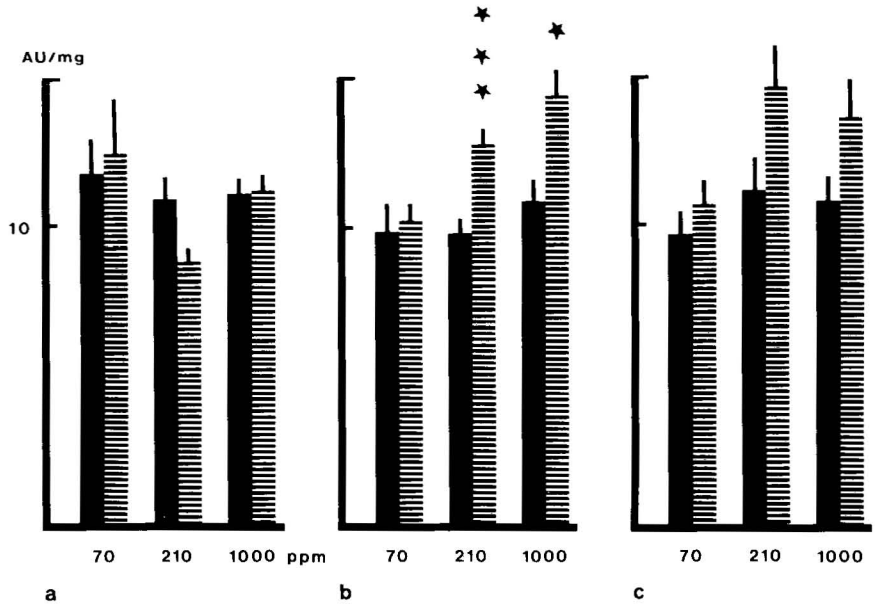

Figure 4. Concentrations of glial fibrillary acidic (GFA) protein per protein in three gerbil brain regions [frontal cerebral cortex (a), sensorimotor cerebral cortex (b), occipital cerebral cortex (c)] after exposure to 1,1,1-trichloroethane at 70, 210, and 1 000 ppm for three months followed by a four-month solvent-free period. The statistical evaluation was performed with Fisher's permutation test (* $p \leq 0.05$, *** $p \leq 0.001$). [Black columns = values for control animals, striped columns = values for exposed animals, AU = arbitrary units (1 AU = amount of GFA protein in 1 μl of the reference gerbil supernatant)]

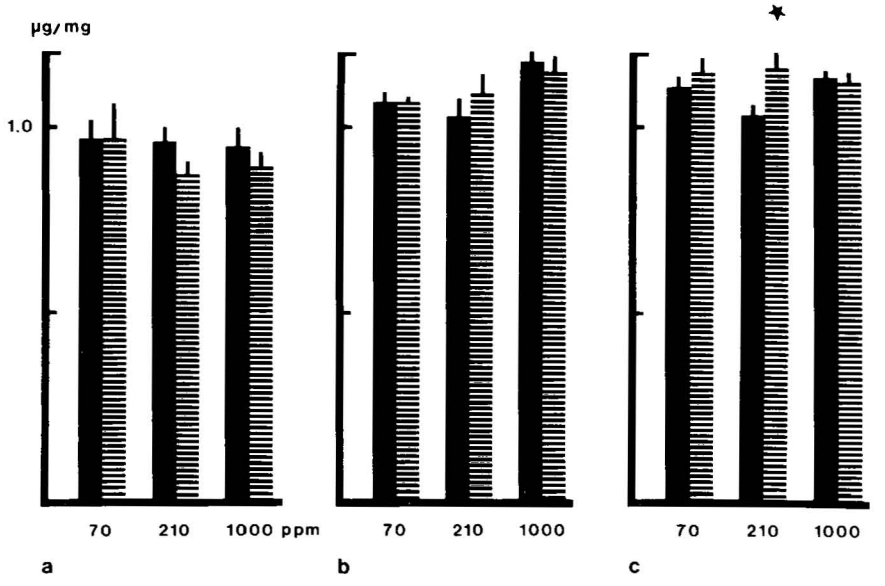

Figure 5. S-100 concentrations per protein in three gerbil brain regions [frontal cerebral cortex (a), sensorimotor cerebral cortex (b), occipital cerebral cortex (c)] after exposure to 1,1,1-trichloroethane at 70, 210, and 1 000 ppm for three months followed by a four-month solvent-free period. The statistical evaluation was performed with Fisher's permutation test (* $p \leq 0.05$). (Black columns = values for control animals, striped columns = values for exposed animals)

ppm, whereas decreased concentrations were found in the occipital cerebral cortex after exposure at 210 ppm (figure 3).

The S-100 and GFA protein concentrations were also calculated as concentrations per protein (figures 4 and 5).

Immunoblotting

Only one band in each sample was found to be GFA protein immunoreactive when the supernatants of the sensorimotor cerebral cortex were subjected to SDS electrophoresis and immunoblotted (figure 6).

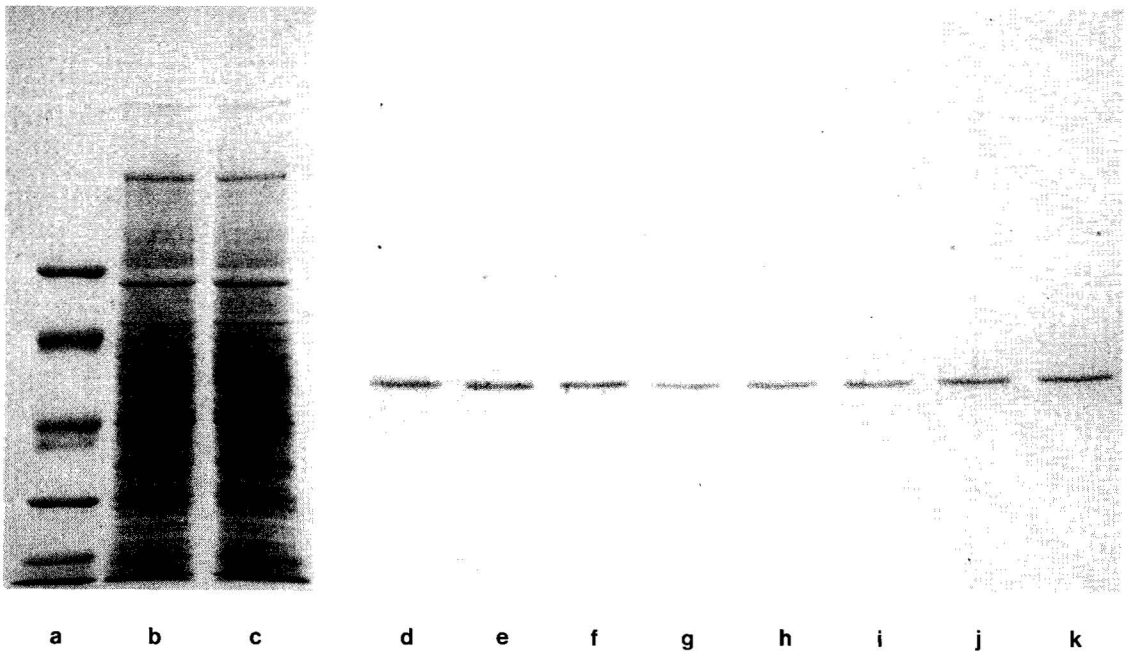

Figure 6. Electrophoretic separation in SDS polyacrylamide gradient gel (5—10 %). Column a: molecular weight standards [from top to bottom: phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soyabean trypsin inhibitor (20 100), α-lactalbumin (14 400)]. Columns b and c: 20 μl of sensorimotor cerebral cortex supernatants (stained with Coomassie brilliant blue) from a control and from a gerbil exposed at 210 ppm, respectively. Columns d-k: electroblott of supernatants from the sensorimotor cerebral cortex of four gerbils exposed at 210 ppm and the four corresponding controls, each calculated to contain five arbitrary units of glial fibrillary acidic (GFA) protein. Columns d, f, h, and j: exposed and columns e, g, i, and k: controls — subjected to electrophoresis as in a, b, and c; the detection was performed with the use of anti-GFA protein diluted 1 :10 000 as the first antibody and swine antirabbit immunoglobulin G conjugated with peroxidase as the second antibody; diaminobenzidine was used for the color reaction.

Discussion

In this study, gerbils were exposed to 1,1,1-trichloroethane by continuous inhalation at 70, 210, and 1 000 ppm for three months. Previous experiments with trichloroethylene and ethanol have confirmed that this length of time is sufficient to induce astroglial cell reactions in gerbil brain (21, 42). After the exposure period, the animals were subjected to a postexposure solvent-free period of four months. This period was included in the experiments to permit an estimation of the lasting or permanent changes induced by the preceding exposure. Earlier experiments have shown four months to be sufficient for neuronal regeneration and restoration of transient astroglial cell reactions after brain injury (7, 8).

Although no adverse effects on the central nervous system were observed in man after chronic exposure to 1,1,1-trichloroethane (24, 35), exposure to other solvents, such as ethanol, toluene and dichloromethane, are known to cause cortical impairment (17, 31, 53). Since long-term exposure to ethanol mainly affects the anterior parts of the cerebral cortex in man (37), different cortical regions were examined in this investigation.

In this study no significant changes in body weight were found between the exposed and control animals. However, a small, but significant reduction of the brain weights was observed among the gerbils exposed at 1 000 ppm when they were compared to control animals. This occurrence may be due to atrophy of the brain at this high exposure level, but other interpretations are also possible, such as dehydration leading to a decreased wet weight.

GFA protein is the main protein subunit of the astroglial filaments and is therefore mainly found in the fibrillary astrocytes (5, 43). Experiments in this laboratory indicate that the extraction procedure used in this study permits solubilization of approximately one-fourth of the total GFA protein in gerbil brain tissue (unpublished observation), and this finding is in agreement with those of Gheuens et al (19). Hence, GFA protein is a suitable marker with which to demonstrate the formation of astroglial fibrils in response to brain injury (4, 10, 29).

Thus the highly significant increased concentrations of GFA protein found in the sensorimotor cerebral cortex after exposure at 210 and 1 000 ppm are compatible with astrogliosis in this brain region. Formation of astroglial fibrils has been demonstrated in several animal models of nervous system injury with the use of the GFA protein as a marker of the astroglial fibrils — for example, stab wounds in the cerebral cortex induces hypertrophy and proliferation of the astrocytes and increased GFA protein content in the damaged zone (4, 29). Wallerian degeneration of the

optic nerve has been shown to induce an increased GFA protein content in the damaged nerve (10). Spinal cord transections (2, 3) and cryogenic lesions of the brain (1), as well as viral and experimental allergic encephalomyelitis (28, 47), are also known to induce formation of fibrils in the astrocytes of damaged tissue. In human neuropathology, the demonstration of astrogliosis is considered indisputable evidence of abnormality in the central nervous system. (See reference 13.) Using antibodies against GFA protein, immunohistochemical studies have, for instance, revealed astrogliosis in postmortem brains of humans with Alzheimer's disease and senile dementia (32).

Hypothetically, the increased concentrations of GFA protein observed could reflect a redistribution between soluble and insoluble forms of the protein, due to either proteolytic degradation (11) or a dissassembly of the glial filaments (43). However, the immunoblott experiment excluded the possibility of proteolytic degradation in the sensorimotor cerebral cortex of the gerbils exposed at 210 ppm. Since there was a significant ($p \leq 0.05$) correlation between the 1,1,1-trichloroethane-induced changes in the concentrations of GFA protein and the other astroglial marker protein used, ie, the concentrations of S-100 and GFA protein changed parallelly, both alternatives seem unlikely. The increased concentrations of GFA protein observed could also reflect a dehydration of the tissue. However, since the changes observed in the sensorimotor cerebral cortex were statistically significant when calculated both per wet weight and per protein (figures 1 and 4), a changed dry : wet weight ratio in the tissue is unlikely.

In brain, the S-100 protein is found in both protoplasmic and fibrillary astrocytes (20). The astroglial localization and the high solubility of S-100 makes this protein a suitable marker for an astroglial cell increase in response to brain damage (7, 38, 40). Previously, astroglial reactions have been demonstrated by increased concentrations of S-100 in gerbil brain after long-term exposure to ethanol, trichloroethylene, and tetrachloroethylene (21, 42, & a paper under preparation by Rosengren et al). Even so, in this study, the increase of S-100 concentrations in the sensorimotor cerebral cortex were not statistically significant after exposure to 1,1,1-trichloroethane at 210 or 1 000 ppm. In view of the differential distribution of S-100 and GFA protein in the two astroglial cell types, this result could at least partly be explained by a shift from protoplasmic to fibrillary astrocytes in response to 1,1,1-trichloroethane exposure at 210 and 1 000 ppm.

1,1,1-Trichloroethane exposure at 210 ppm seems to induce decreased concentrations of the S-100 in the frontal cerebral cortex, as calculated per wet weight but not per protein. This occurrence could imply a changed dry : wet weight ratio and a changed protein content in this brain region. An 1,1,1-trichloroethane-induced degeneration of the astroglial cells in this brain region is also a possible explanation. Another chlorinated olefin, tetrachloroethylene, has previously been shown to decrease the S-100 concentrations in this region of the gerbil brain after similar exposure at 320 ppm (paper under preparation by Rosengren et al). However, in this study, after exposure at 1 000 ppm, the decrease in the S-100 concentrations was not statistically significant in this brain region. Hence, the rationale for the decreased S-100 concentrations after exposure to 1,1,1-trichloroethane at 210 ppm is unclear.

The mechanism behind the neurotoxicity of 1,1,1-trichloroethane is not known. In view of the negligible metabolism of 1,1,1-trichloroethane (45, 46), the low content of the cytochrome P-450 monooxygenase system in rat brain (34), and the chemical stability of the postulated metabolites (23), local biotransformation in the central nervous system is probably of too little importance to explain the neurotoxicity of the solvent. In a study of the uptake and release of 1,1,1-trichloroethane in the brain of rats, no detectable metabolites were found (54). On the other hand, as 1,1,1-trichloroethane is lipid-soluble (44) and as high concentrations of the solvent can be reached in the central nervous system during exposure (54), effects on brain membranes are probable. Previously, the polyunsaturated fatty acids of ethanolamine-phosphoglyceride have been shown to be altered in gerbil brain after long-term exposure to both trichloroethylene and tetrachloroethylene; this phenomenon probably reflects a compensatory mechanism for the fluidizing effects of the solvent on brain membranes (25, 26).

The biochemical alterations induced after chronic exposure to 1,1,1-trichloroethane demonstrate a pronounced change in gerbil brain. These alterations are compatible with astrogliosis in the cerebral sensorimotor cortex. It is suggested that astroglial reactions are a common feature after long-term exposure to some solvents (21, 42), as well as to 1,1,1-trichloroethane, and that the changes observed are lasting or irreversible, as they can be found after a postexposure solvent-free period. Thus, 1,1,1-trichloroethane should not be considered harmless, as has been previously claimed (49).

Acknowledgments

This work has been supported by the Swedish Work and Environmental Fund (ASF 82-0649, ASF 83-0810 and ASF 84-0217).

Ms E Åwall's skillful technical assistance is gratefully acknowledged, as is the statistical advice of Mr L Trulsson, of Göteborg's Datacentral.

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Received for publication: 11 June 1985