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A morphometric evaluation of the effects of trichloroethylene and dichloroacetylene on the rat mental nerve. Preliminary results

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Morphometric analysis was used to compare the effects of trichloroethylene (Tri) and dichloroacetylene (Dca) on the fibre parameters of the trigeminal nerve. Treated animals were clearly separated from controls according to a discriminant analysis. Furthermore, in the class of nerve fibres defined by a clustering analysis and corresponding to the largest fibres, myelin thickness was significantly decreased in the Dca group, but less so in the Tri group. In the group of the smallest fibres however, the myelin thickness was significantly increased by the treatments, but especially by Tri. Such a variability in the effects of Tri has already been demonstrated. Mechanisms for this are quite unclear although demyelination could be involved as already suggested. Our results thus show the ability of Tri and Dca to alter nerve parameters but probably with different modes of action depending on the size of the fibre.

Neuropsychological manifestations and impairment of the cranial nerves are prominent features of trichloroethylene (Tri) exposure [17, 22]. Several experimental studies have tried to characterize the lesions of the most affected nerve: the trigeminal nerve [1–4, 10]. The results were not convincing and dichloroacetylene (Dca), a breakdown product, has been suspected on the basis of clinical and experimental reports [9, 14]. Nevertheless, the discussion is still open because of the wide range of modes of exposure, animals, doses and because of different techniques for tissue fixation and analysis which do not avoid postmortem artefacts. A viral hypothesis has even been proposed in preference to a direct biochemical mode of action on the nerve of these compounds [5]. Whatever the mechanism for nerve alterations, no comparative study of Tri versus Dca has ever been done under the same experimental conditions.

Having this in mind, our study aimed at describing the effects of Tri and Dca on the trigeminal nerve, by the use of morphometric analysis which is a useful tool for assessing nerve alterations or for developmental studies of the peripheral nerve system [6, 18, 19]. This analysis was performed in parallel for Tri and Dca in conditions where Dca formation from Tri was particularly improbable.

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Twenty-one female Sprague–Dawley rats (53 days old) weighing between 210 and 245 g were randomly divided into 3 separately caged groups, with food (commercial preparations) and water ad libitum.

The animals were intoxicated by direct gastric administration in an oil suspension once a day, 5 days per week for 10 weeks. The doses were 2.5 g/kg for Tri and 17 mg/kg for Dca according to the ratio between the LD_{50} of the two substances. Dca was synthesized according to Siegel et al. [15] and Tri obtained from Prolabo (R.P. Normapur for analysis).

This method gave better control of the toxic administration compared to inhalation, and eliminated local toxicity through the mucosa of the upper respiratory tract. This mode of intoxication also rends improbable Dca formation from Tri as while several experimental findings suggest formation of Dca when Tri comes into contact with moderate alcaline material, none suggests that a metabolic formation could occur.

The tolerance to the exposure was estimated by the evolution of body weight, food and water consumptions. The animals were anaesthetized (phenobarbital given intra-peritoneally, 50 mg/kg), and the mental nerve was dissected and then immerged in the fixative for at least 3 h (2.5% glutaraldehyde in a 0.2 M sodium phosphate buffer, pH = 7.4), washed 8 h in buffer, post-fixed in osmium tetroxide for 90 min, dehydrated and embedded in Epon.

All measurements were made on the mental nerve, a superficial branch of the trigeminal nerve, chosen because its fibre density is compatible with an automatic morphometric analysis.

The main nerve fibre parameters (i.e. external and internal diameters, myelin thickness) were calculated on silver-stained transverse semithin sections (1 μm) [16] by an automatic morphometric analysis. We used a program developed in C language on a Samba^{TM} 2005 cell image analysis system (Alcatel-TITN Answare, Grenoble, France) equipped with a position control system MCC (Märtzhäuser, Wetzlar, F.R.G.) to monitor the microscope stage displacement and the fine focus knob. Fibre segmentation was made according to the procedure of adaptive grey level thresholding which gave the most reliable results. Fibre clusters were autonomically separated using a labelling algorithm and in the case of failure, the mask of the fibre was shown to the operator who could then delimit the contour of the fibre to be analysed by means of interactive graphic tools. For further details, see ref. 21.

Variances and mean comparisons were first classically made by ANOVA. However, the multi-modal distribution of the nerve fibre parameters (Fig. 1) rends ANOVA somewhat unappropriate. We therefore used the moving means clustering method which first consists in a principal component analysis (PCA) to normalize the data and then in a clustering analysis on the space defined by PCA to obtain classes of nerve fibres. The stability of these classifications was verified by the repetition of the cluster analysis. A stepwise discriminant analysis (SDA), was then applied to stress the differences between experimental and control groups (see ref. 20 for more details). Finally, ANOVA was made to specify the nature of these differences.

Clinical changes. According to one factor ANOVA-repeated measures, the influence of time on the body weight was significant in all groups ($P<0.001$). Whereas no difference was observed between animals within the control group ($F=2.207$, $P=0.06$), significant differences were found within each treated group, thus sug-

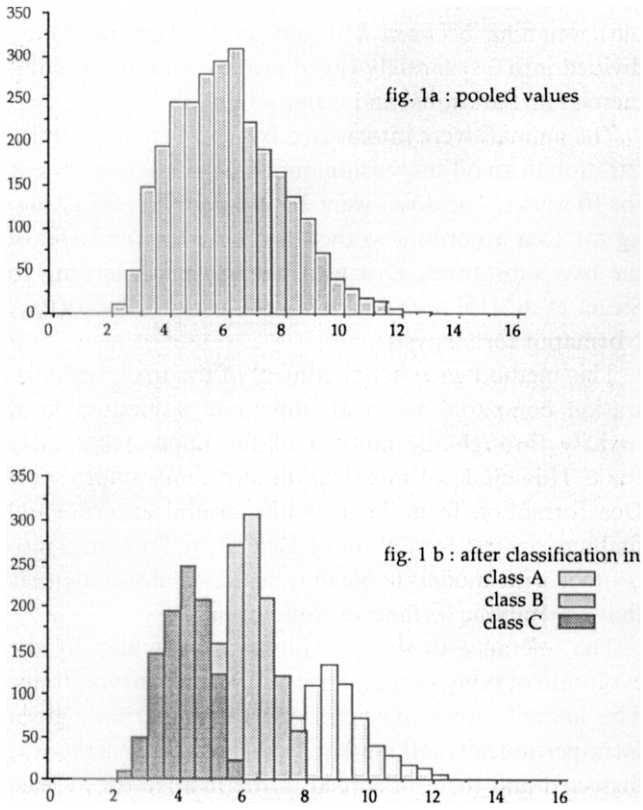

Fig. 1. Histograms of external diameter in the control group ((a) pooled values and (b) after classification in 3 classes by the moving means clustering method applied to the fibres measured in each rat and then pooled together within each group of animals).

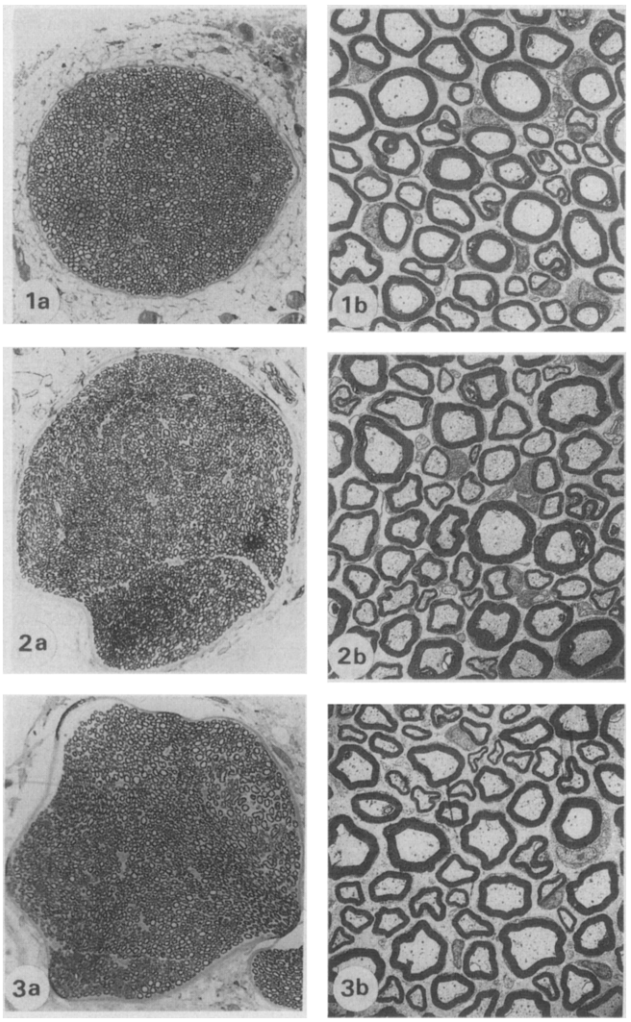

Fig. 2. Nerve samples under light (a) and electron (b) microscopy showing on one hand the extreme high density of the nerve fibre and on the other hand the good quality of the preservation of the myelin sheaths and axons of the nerve samples. 1: control rat. 2: Tri intoxicated rat. 3: Dca intoxicated rat. a: × 160. b: × 1400.

gesting the effectiveness of the treatment (within Tri group $F=9.51$, $P<0.001$; within Dca group $F=13.22$, $P<0.001$). Furthermore, the mean weight gain at the end of the period of exposure was significantly reduced in Dca rats (Dca = 24.71 g, S.D. = 15.3; Controls = 60.8 g, S.D. = 15.8; $F=19.86$, $P<0.05$), but not in the Tri group (Tri = 55.5 g, S.D. = 19, N.S.). Finally, no significant difference was observed in the mean food and water consumptions between the 3 groups, a fact which does not favour the hypothesis of a simple effect of undernourishment on the weight.

Histopathological examination. No significant structural modifications were visible by light and electron microscopy in any of the nerves examined after intoxication (Fig. 2). This figure also shows on the one hand the extreme high density of the nerve fibre and on the other hand the good quality of the preservation of the myelin sheaths and axons of our nerve samples.

Results of the morphometric evaluation. The values of the nerve fibre parameters measured in each rat of the same group were pooled together and analyzed by ANOVA. Significant differences in their variances as well as in their means were obtained (see Table I).

Means in the Tri group were surprisingly increased compared to controls whereas they were decreased in the Dca group, although a similar trend in the two treated groups was expected since Dca, a breakdown product of Tri, was supposed to act in the same way as Tri.

Nevertheless, large inter-individual variations in the mean values of the parameters were especially observed in the treated groups and with a lesser extent within the control groups, as 90% of the different arrangements in pairs of the animals differed within the Tri group, 93% in the Dca group against 62% within the control group (ANOVA). These variations can be either due to intrinsic factors such as weight influence, anatomical or sampling variations or to extrinsic factors such as a variable individual sensitivity to a toxic exposure as these variations were considerably higher in the treated groups. The influence of the heterogeneity of the frequency distribution of the values of the parameters must also be considered (Fig. 1). All these facts justify the use of a multiparametric analysis described in the text.

According to the moving means clustering method, a classification in 3 classes was the most consistent; they were called A, B, C with mean values of the external diameter ranging from A to C in a descending order (Fig. 1).

According to a stepwise discriminant analysis, the experimental animals were clearly separated from controls: all the Dca rats and 6 out of 7 Tri rats were correctly classified, only 1 Tri ra[illegible] was classified with controls. The most discriminating parameters were successively the internal diameter of class A then of B, the exter-

TABLE I

MYELINATED FIBRE PARAMETERS

Class A, B and C were obtained after classification in 3 classes by the moving means clustering method applied to the fibres parameters measured in each rat and then pooled together within each group of animals to be studied by ANOVA. All values are exposed in micrometers.

	Number of fibres	External diameter (mean ± S.D.)	Internal diameter (mean ± S.D.)	Myelin thickness (mean ± S.D.)
Control group				
Pooled values	2782	6.006 ± 1.84	3.863 ± 1.40	0.959 ± 0.30
class A	615	8.596 ± 1.09	5.765 ± 0.95	1.314 ± 0.26
class B	1160	6.222 ± 0.70	4.005 ± 0.68	1.000 ± 0.19
class C	1007	4.171 ± 0.74	2.537 ± 0.62	0.693 ± 0.16
Tri group				
Pooled values	2915	6.206* ± 1.92*	4.004* ± 1.48*	1.004* ± 0.35*
class A	755	8.544 ± 1.09	5.721 ± 1.15*	1.340 ± 0.33*
class B	1148	6.325* ± 0.96*	4.092* ± 0.85*	1.018 ± 0.25
class C	1012	4.857* ± 1.56*	3.012* ± 1.23*	0.813* ± 0.25*
Dca group				
Pooled values	2528	5.872* ± 1.97*	3.793 ± 1.47	0.931* ± 0.33*
class A	486	8.659 ± 1.61	5.927* ± 1.15*	1.272* ± 0.35*
class B	1009	6.248 ± 1.02*	4.042 ± 0.73*	0.993* ± 0.27*
class C	1033	4.192 ± 0.80	2.546 ± 0.62	0.710 ± 0.20*

*$P<0.05$ (variances and means were compared to the matched control group, by ANOVA).

nal diameter of class A then of B and finally the myelin thickness of class A and then of C, the final percentage of missclassification was 5% (parameters were classified as a function of their decreasing discriminatory power, but it must be said that none of them had enough discriminatory power to support a correct classification in itself).

To specify these modifications, all the fibres classified by the moving means method in each rat were pooled together within each group of animals and then studied by ANOVA.

In such conditions, the variations mainly consisted of significant differences in the variances of the parameters and also, to a lesser extent, in their mean values (Table I). It must be thus pointed out that there was a tendency for the medium sized and the smallest fibres in the Tri group to have increased diameters and myelin thickness whereas diameters in the largest fibres were rather decreased. In the Dca group, there was also a tendency for increased diameters in the smallest fibres but the striking feature is the observation of a significant decrease in the myelin thickness especially in the largest and in the medium-sized fibres (see Table I). The most significant parameters identified by ANOVA were strictly not superposable to those identified by the multiparametric analysis. This was due to the different nature of these tests and to the fact, already mentioned, that the discriminatory powers of the parameters were very close.

Some modifications of the nerve fibre parameters due to the treatment can be thus identified despite the inter-individual variations previously mentioned. The observation of decreased myelin thickness in the Dca group and the reduced diameter in the Tri group could suggest demyelination, an effect already observed in other brain areas [11, 12]. Recent evidence for the production of free radicals mainly from trichloroethanol, a metabolite of Tri, could account for this process [7]. On the other hand, in the smallest fibres, the myelin thickness is significantly increased in the Tri group and also slightly in the Dca group. The reasons for this variability in the effects depending on the size of the nerve fibres are quite unclear but this variability has already been observed in other brain areas [8, 12, 13].

Our results thus show that after Tri or Dca exposure significant modifications of nerve fibre parameters between treated animals and controls are observed and that the characteristics of these modifications even seem to vary with the nature of the toxic agent, possibly suggesting different modes of action.

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