An ultrastructural study of neuronal changes in dorsal root ganglia (DRG) of rats after chronic cisplatin administrations

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Summary. In humans, the main dose-limiting side-effect of cisplatin (CDDP) treatment is a peripheral sensory neuropathy secondary to dorsal root ganglion (DRG) neuron involvement. To investigate further for neuronal alterations responsible for CDDP neurotoxicity we undertook the present experimental ultrastructural study, based on observations of 3 different groups of rats (6 animals in each group). Group A rats were treated with 1 mg/kg weekly for 9 weeks; Group B with 2 mg/kg weekly for 9 weeks; and group C rats served as untreated controls. At the end of the experiment, rats were perfused with 3% glutaraldehyde and lumbar DRGs were prepared for electron microscopic observations. In CDDP-treated rats somatic, nuclear and, above all, nucleolar size was reduced. Ultrastructurally, the nucleolus was the most affected structure. Nucleolar alterations were quantified morphometrically. Less marked changes were seen in the nucleus and in the RER and Golgi apparatus of the cytoplasm. The number of lysosomes and lipofuscins was greatly increased in CDDP-treated rats.

The ultrastructural alterations observed in CDDP rats suggest that CDDP may be neurotoxic due to a reduction in protein synthesis. This assumption would explain why cells such as neurons, which are non replicating, but which have a high rate of protein synthesis, may be the target of the neurotoxic action of CDDP. The lack of an efficient blood/nerve barrier in the DRG explains the involvement of this particular type of neuron.

Key words: Cisplatin, Dorsal root ganglia, Neuropathy, Ultrastructure

Introduction

Cisplatin (cis-diamminedichloroplatinum II, CDDP) is widely used in clinical practice to treat solid tumors

(Rosenberg, 1985), but it also has serious side affects, which limit its clinical use. The major cytotoxic side-effect induced by CDDP is sensory peripheral neuropathy (Von Hoff et al., 1979; Carter, 1984; Cavaletti et al., 1992a). In experimental animals it has been demonstrated that the peripheral neuropathy is secondary to the pathological involvement of dorsal root ganglion (DRG) neurons (Tomiwa et al., 1986; Müller et al., 1990; Cavaletti et al., 1991, 1992b). Determinations of CDDP content in nervous tissues have shown that the highest concentrations are found in dorsal root ganglia in rat (Cavaletti et al., 1990) and humans (Gregg et al., 1992) while in the central nervous system CDDP content is very low, indicating an efficient blood-brain barrier to the drug (Thompson et al., 1984; Cavaletti et al., 1990).

The therapeutic effect of CDDP is due to its interaction with DNA (Roberts and Thomson, 1979; Lippard, 1982). In fact, covalent binding of CDDP to DNA leads to the inter- and intra-strand cross-links (Eastman, 1983; Fichtinger-Schepman et al., 1985; Pinto and Lippard, 1985b; Eastman and Schulte, 1988) which inhibit DNA replication (Harder and Rosenberg, 1970; Howle and Gale, 1970; Salles et al., 1983; Pinto and Lippard, 1985a; Ciccarelli et al., 1985).

The inhibition of DNA replication does not explain CDDP-induced neurotoxicity, since DRG neurons are not mitotic cells and no DNA synthesis occurs in these cells. Other mechanisms have been suggested to explain CDDP-induced neurotoxicity. In fact, previous studies have indicated that the nucleolus (where synthesis and processing of preribosomal RNA and assembly of proteins into preribosomal particles take place) is the structure most affected in CDDP neuropathy (Tomiwa et al., 1986; Cavaletti et al., 1992b). Furthermore, extensive binding of CDDP within the cytoplasm has also been reported (Parti and Wolf, 1990), indicating that the cytotoxic effect of the drug may not be limited to the nucleus alone but may also involve the cytoplasm. A number of in vitro studies have demonstrated that CDDP is able to bind to nucleophiles such as proteins and RNA (Levi et al., 1980; Rosenberg, 1985; Hegedüs et al., 1987; Tay et al., 1988).

With this background in mind, the aim of the present study was to extend previous observations regarding the toxic effects induced by CDDP on DRG neurons in rats, in order to clarify the main neuronal alterations responsible for CDDP neurotoxicity. This goal can be achieved only by using morphological techniques (mainly ultrastructural), since electrophysiological determinations give no clues as to the overall alterations affecting DRG neurons, nor do they localize the cellular sites involved in the pathological processes.

Materials and methods

Experimental model

18 female Wistar rats, weighing approximately 180 g at the beginning of the experiment, were used in the present study. They were individually housed in plastic cages and given commercial rat cubes and tap water ad libitum.

Lyophilized CDDP (Platamine, Carlo Erba Farmitalia, Italy) was dissolved in sterile saline solution and administered at a dosage of 1 mg/kg weekly (6 rats, group A) and 2 mg/kg weekly (6 rats, group B) for 9 weeks. Control rats (6 rats) were treated with sterile saline solution only. The CDDP solution was injected intraperitoneally, immediately followed by 3 ml of sterile saline solution given subcutaneously to prevent renal damage due to hyperhydration. 10 mg of Mannitol was added to increase diuresis. CDDP was always administered between 12.00 am and 2.00 pm.

Pathological examination

At the end of the experiment the animals were killed under chloral anaesthesia by intracardiac perfusion with 3% cold glutaraldehyde in 0.12M phosphate buffer solution, and the lumbar spinal ganglia L4-L6 were removed. All animals were killed between 12.00 am and 2.00 pm to prevent circadian nucleolar modifications (Fakan and Hernandez-Verdun, 1986).

The ganglia were washed in 0.18M phosphate buffer solution, postfixed in 1% OsO_4, dehydrated and embedded in epoxy resin. Ultrastructural observations were performed with a Philips CM 10 electron microscope. Morphometric determinations were performed with an automatic image analyzer (TAS Plus,

Leica GmbH) on toluidine blue-stained semithin sections (1 µm). Cross-sectional somatic, nuclear and nucleolar area, and the number and eccentricity of the nucleolus(i) were determined (in at least 300 neurons for each animal). The percentage of the sectional area of the different nucleolar components was determined in at least 25 randomly-selected nucleoli for each group using electron microscope micrographs at a final enlargement of x80,000. The percentage of the cross-sectional area of the nucleolus occupied by the vacuolar (interstitial) component and the granular component were determined with the image analyzer. All the other components, such as the condensed chromatin and the fibrillar component, which are less easily separated than the previous components, were reported as a single component, the value of which was obtained by calculating the difference between the total nucleolar area and the sum of the previous two components.

Results

Histological morphometric determinations

Morphometric data are summarized in Table 1. The

Table 1. Morphometric data.

	CONTROLS	GROUP A	GROUP B
Somatic area (µm²)	951	751*	753*
	(895-1006)	(708-795)	(711-795)
Nuclear area (µm²)	147.6	122.7*	117.7*, **
	(141.9-153.3)	(117.9-127.4)	(106.0-117.4)
Nucleolar area (µm²)	11.21	9.76*	7.43*, **
	(10.53-11.89)	(9.56-9.96)	(7.15-7.71)
Multiple nucleoli	3.3%	6.8%	14.1%
Eccentric nucleoli	32.3%	30.8%	51%

*: p<0.01 referring to controls; **: p<0.01 referring to group A.

Fig. 1. Electron micrograph of a nucleolus of a CDDP-treated rat characterized by segregation of the fibrillar and granular components of the nucleolus and formation of a large fibrillar centre (arrows) surrounded by dense fibrillar component (arrowheads). Granular component is reduced (open arrows). Bar= 0.4 µm.

most striking changes occurred in the nucleoli of DRG neurons, but significant differences in nuclear and somatic area were also present. The mean nucleolar area was significantly reduced in treated rats with respect to the controls. Moreover, the nucleolar number per nucleus and nucleolar eccentricity also increased significantly in treated rats. The extent of these alterations was greater in rats which received the higher dose of CDDP.

The mean somatic and nuclear area were also reduced The degree of reduction in the somatic area was similar in both groups of treated rats, whereas the
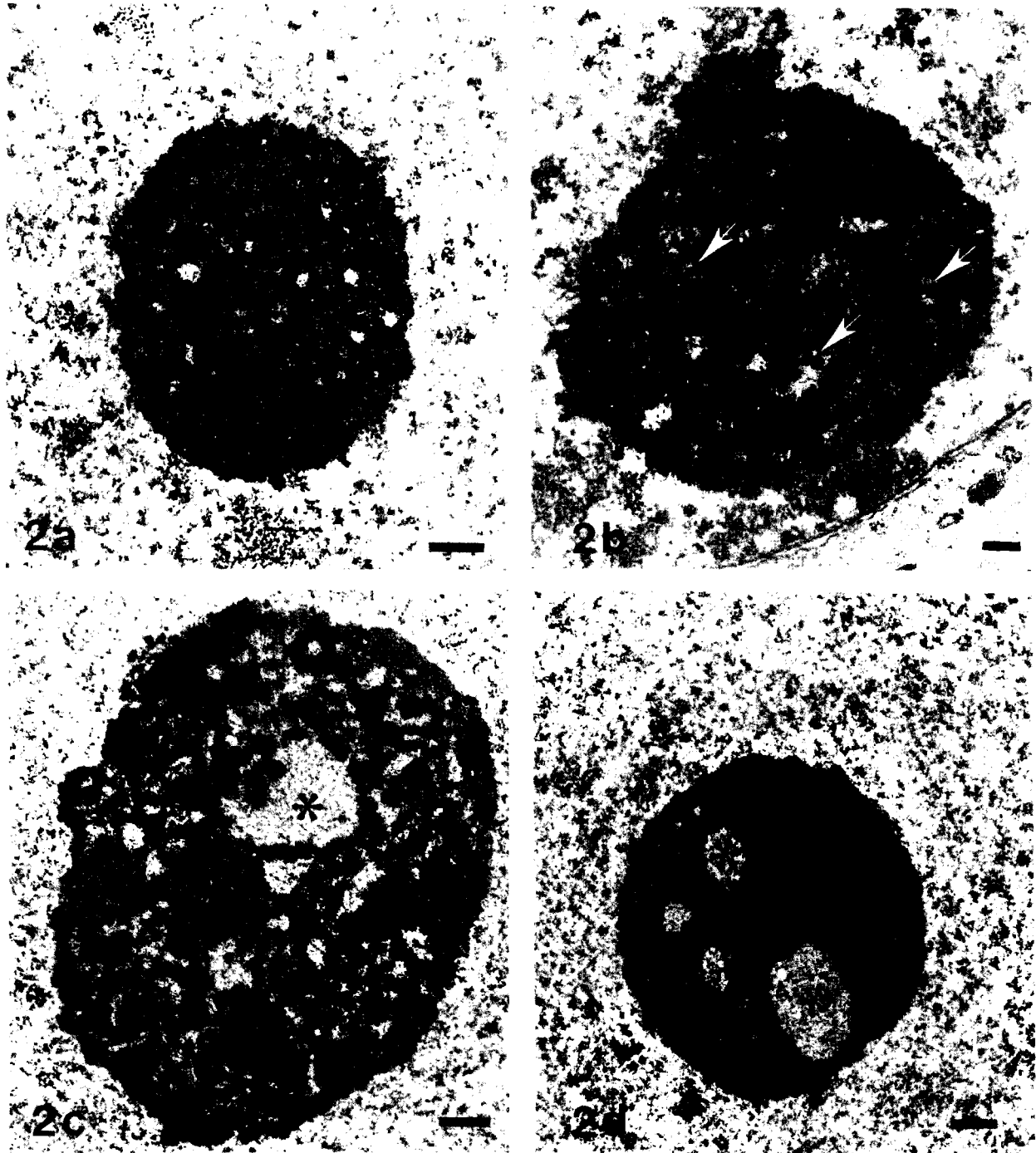

Fig. 2. Electron micrographs of the nucleolus. a. Normal nucleolus. b. Microspherules (arrowheads) in the intranucleolar interstices. c. Segregation of the interstices to form a large central vacuolus (asterisk). d. Formation of few large interstitial vacuoli (asterisks). Bars: a, 0.5 µm; b, 0.2 µm; c, 0.5 µm; and d, 0.5 µm.

reduction in the mean nuclear area was again more evident in rats which underwent the more intense CDDP treatment.

Electron microscope changes

Ultrastructural alterations were particularly striking in the nucleolus, but the nucleus and cytoplasmic organelles also appeared to be involved.

Nucleolar changes

A common and striking alteration in the nucleoli was a segregation of the granular from the dense fibrillar component and the formation of large fibrillar centres (Fig. 1, compare with Fig. 2a). Segregation was present in 20% of the nucleoli in group A, in more than 40% of the nucleoli in group B compared with only 5% in the control group.

Some of the nucleoli in the treated rats had lost their nucleolonemal integrity and their roundish profile. These nucleolar alterations were again more evident in rats which received higher doses of CDDP and were commonly found in those nucleoli that also exhibited segregation of the nucleolar components.

Microspherules (composed of fibrillar material) were only occasionally observed in some intranucleolar interstices in control rats, while they were quite common in the nucleoli of DRG neurons of treated rats (Fig. 2b) which also showed an increase in the size of the interstices (see: quantitative nucleolar data). Occasionally, nucleoli of group B rats showed a few large and prominent interstitial vacuoli, which sometimes occurred singly and were located at the centre of the nucleolus, as the result of a process of «segregation» of the nucleolar interstices (Fig. 2c,d).

Nuclear changes

In CDDP-treated rats the nucleoplasm was generally less condensed than in the controls. Zones of chromatin disruption, i.e. a «clearing» of the nucleoplasm (Tomiwa et al., 1986), could be observed (Fig. 3). The increased number of nuclear membrane infoldings in CDDP-treated rats, reported by Tomiwa et al. (1986) and Cavaletti et al. (1991a, 1992b) on the basis of qualitative evaluations, was not substantiated by a quantitative determination.

Cytoplasmic changes.

The most evident alteration was a reduction and fragmentation of the rough endoplasmic reticulum (RER) in group B rats. This change was sometimes associated with an increase in membranes deprived of ribosomes and with a decrease in the number of free ribosomes and polisomes (Fig. 4b). These changes were more evident in large «pale» neurons. Shrinkage of the blocks of RER was very often associated with a widening of the neurofilamentous bands (Fig.
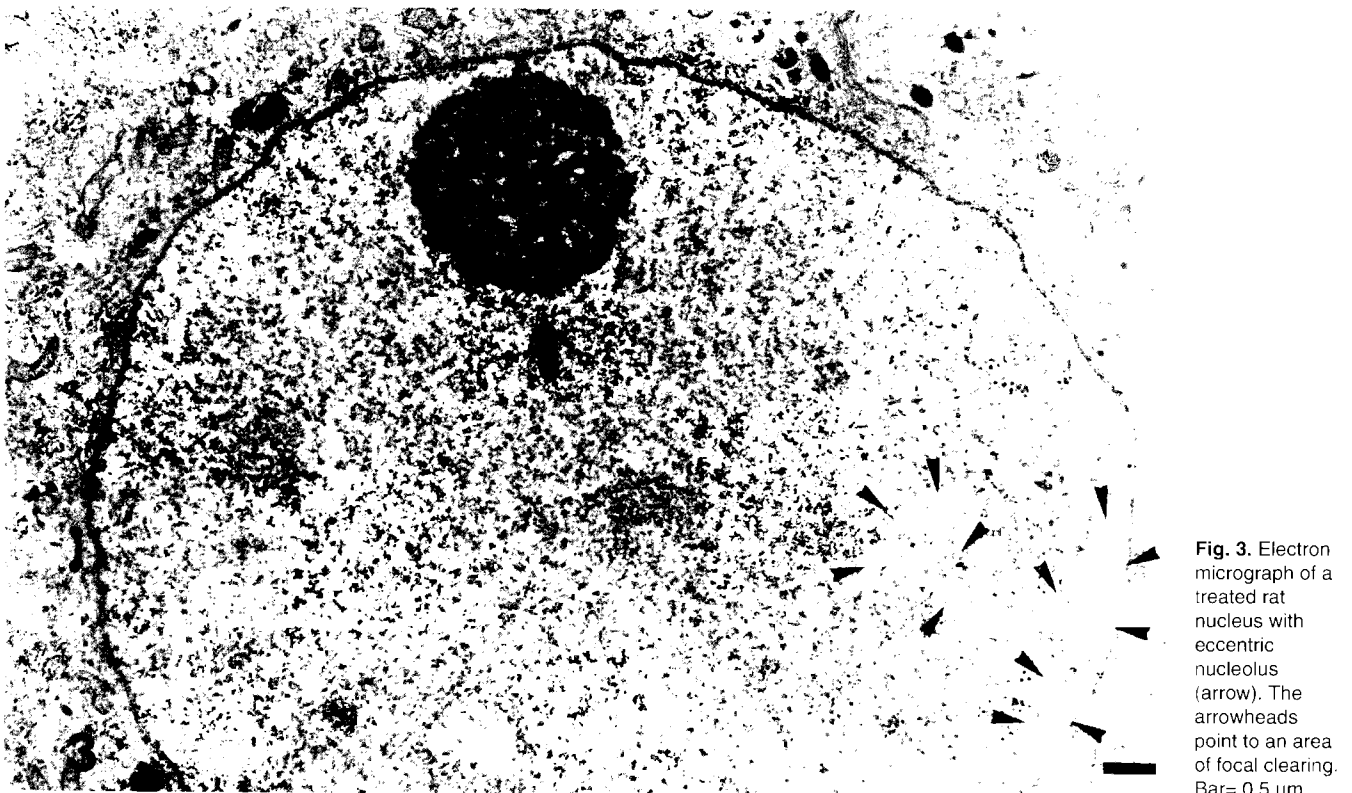

Fig. 3. Electron micrograph of a treated rat nucleus with eccentric nucleolus (arrow). The arrowheads point to an area of focal clearing. Bar= 0.5 μm.

4b).

The Golgi apparatus was unaffected in group A. However, in group B single dyctiosomes were reduced in size and had lost their typical concave morphology, while the flattened cisternae appeared dilated (Fig. 4a). Mitochondria were mildly affected only in group B. A few mitochondria were swollen and their matrix was less dense. There was, however, an increase in the number of
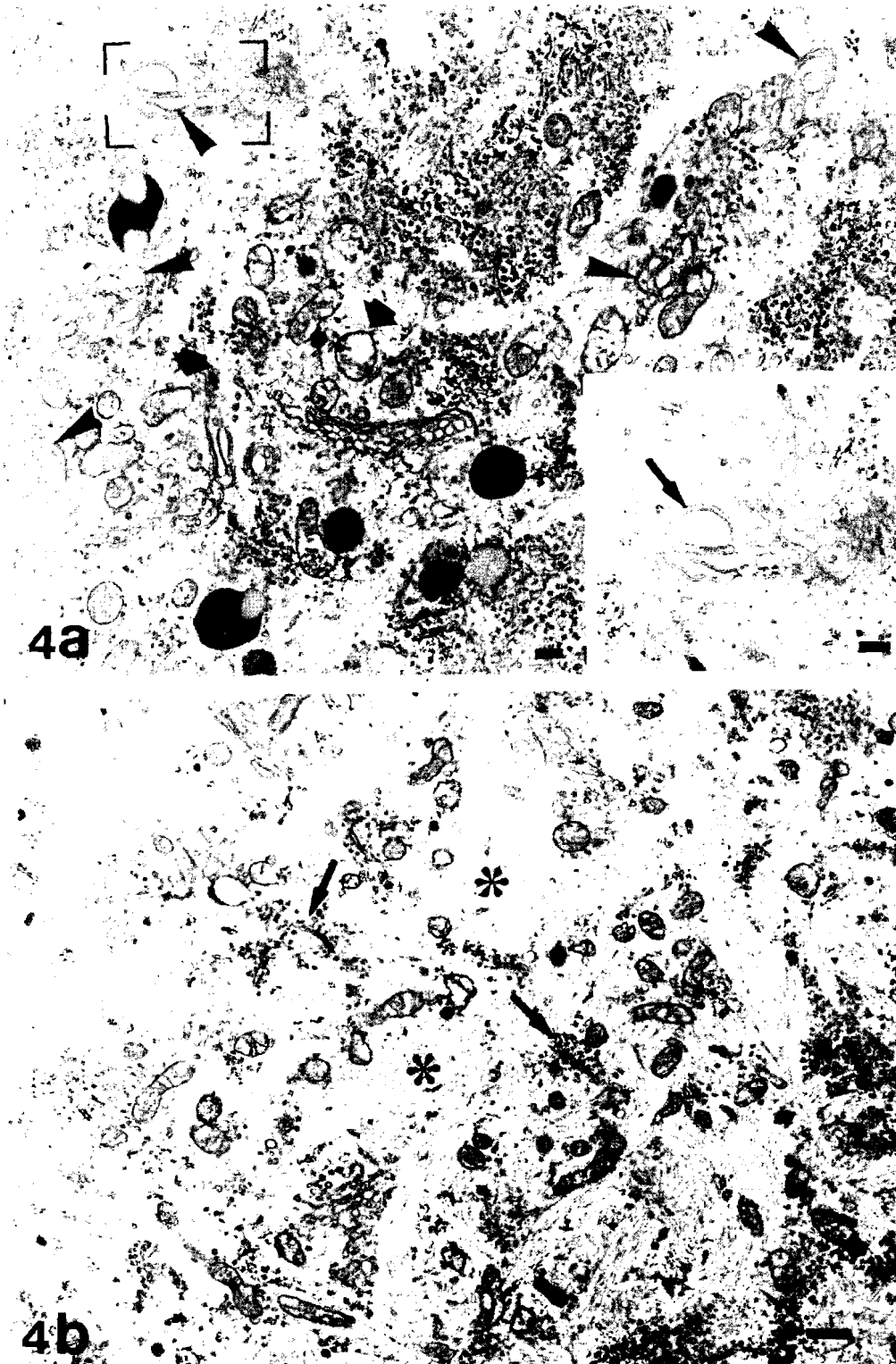

Fig. 4. Electron micrographs of the cytoplasm of treated rats. **a.** Dilatation of the cisternae of the Golgi apparatus (arrowheads) and swollen mitochondria (arrows), shown at higher magnification in the inset. **b.** Reduction and fragmentation of the rough endoplasmic reticulum (RER) (arrows). The neurofilaments are increased and form bands (asterisks) between the RER blocks. Bars: a, 0.25 μm; b, 0.5 μm; inset, 0.2 μm.

lysosome-like structures in CDDP-treated rats, particularly in large «pale» neurons. This fact was already apparent at light microscope level, the lysosomes being visible as small black dots (Fig. 5b). At electron microscope level, lysosomes of treated animals sometimes showed small electron-dense inclusions (Fig. 5a) and they had often lost their regular profile. In a few cases large lysosomes were observed in close contact with abnormal clumps of cytoplasmic organelles, mainly mitochondria, but also incomplete lamellae of the RER and Golgi cisternae. The increase in lysosomes was associated with a proportional increase in lipofuscins.

Finally, in group B rats, a few neurons underwent «watery degeneration» of the hyaloplasm (Fig. 5c).

Quantification of nucleolar changes

Morphometric analysis of the distribution of the different nucleolar components revealed that significant changes in the nucleolar ultrastructure had taken place (Fig. 6). The total area occupied by the interstitial (or vacuolar) component increased significantly in treated animals. The increasing of the interstitial area was associated with a decrease in the granular component. The percentage of the area occupied by the interstitial component increased significantly in both groups of treated rats. The increasing of the interstitial component of the nucleolus was much greater than the decrease in size of the nucleolus in treated rats. Changes in the interstitial component were, therefore, real and were not the result of a concomitant reduction in the overall
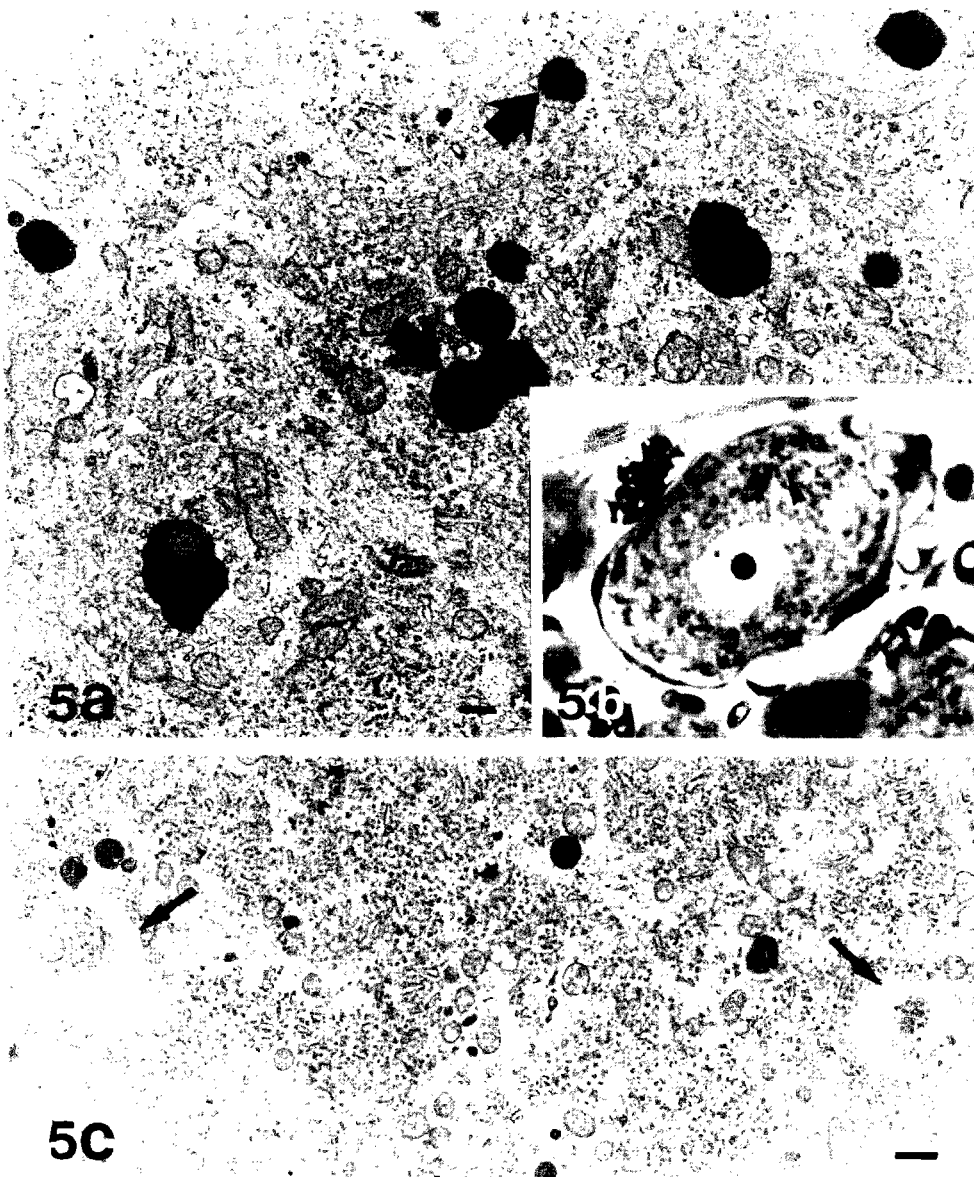

Fig. 5. a. Electron micrograph demonstrating the variable size and appearance of the lysosome-like structures. Arrows point to small electron-dense inclusions. b. Light microscope micrograph of a large pale neuron with increased number of lysosome-like structures which appear as small black dots. c. Watery degeneration of the hyaloplasm (arrows). Bars: a, 0.25 μm; b, 5 μm; c, 0.5 μm.
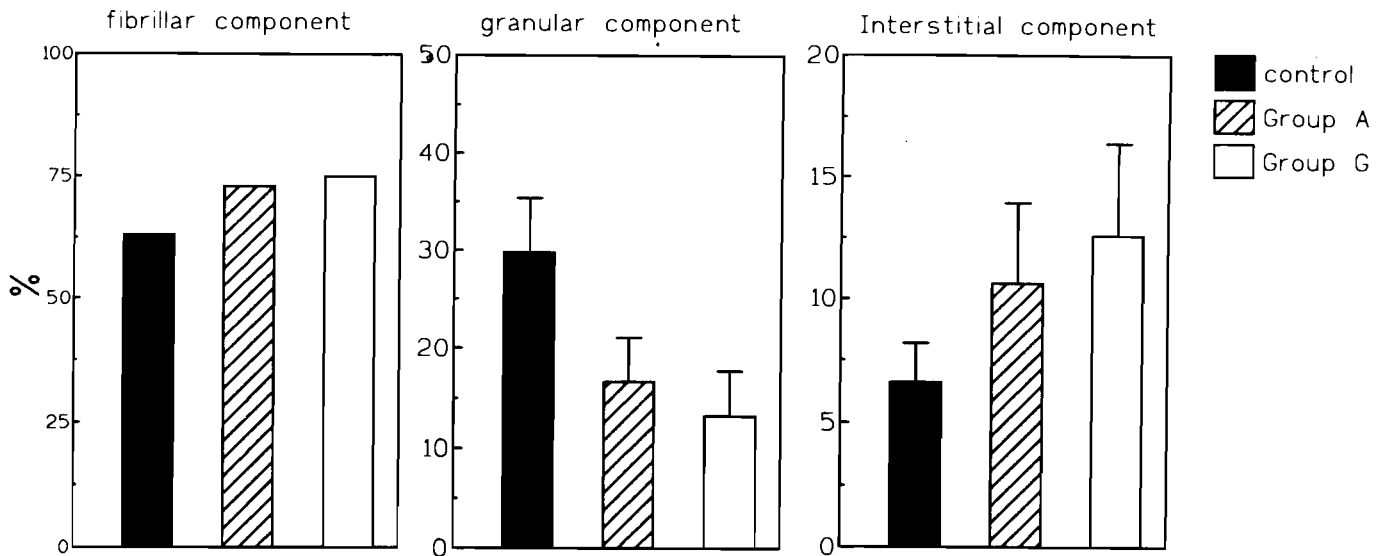
Fig. 6. Histograms of the percentage of the nucleolar area occupied by the different nucleolar components.

nucleolus size.

The increase in the interstitial component was concomitant with a significant decrease in the granular component. The fibrillar component increased in a similar way in both treated groups. The increase was mainly due to the occurrence of large fibrillar centres (Fig. 1), which were considered under this heading.

It was difficult to determine the actual degree of these nucleolar changes, since the overall nucleolar size, to which the percentages of the different components are related, also decreased considerably and, therefore, may have masked a greater alteration of the different nucleolar components than was apparent.

Discussion

The present study demonstrates that DRG neurons undergo pathological changes after chronic systemic CDDP administration. The degree of the different pathological changes is dose related and the nucleolus is the cellular site most affected by CDDP administration.

The nucleolus is an active genetic site where synthesis and processing of preribosomal RNA take place together with the assembly of proteins into the preribosomal particles. Any change in nuclear morphology is closely related to variations in nucleolar activity (Lafarga et al., 1991). In this context CDDP-induced nucleolar changes (such as nucleolar atrophy, segregation of the nucleolar components, increase in vacuole size, decrease in the granular component, increasing of the microspherules) may all be indicative of a decline in nucleolar function, i.e. a reduction in the nucleolar transcription rate and/or a decrease in the ability to process and assemble pre-rRNA in pre-ribosomal particles (for review see Goessens, 1984). This assumption is supported by three main pieces of

evidence: 1) actinomycin, which inhibits the transcription of nucleolar genes (Arrighi, 1967; Recher et al., 1971) induces nucleolar segregation; 2) drugs which stimulate nucleolar transcription activity, such as thioacetamide, induce changes diametrically opposed to those observed in our experiment, namely an increasing of the granular component and a loss of interstitial spaces (Moreno Diaz de la Espina et al., 1980; Franke et al., 1981); and 3) microspherules have been interpreted as being immature pre-ribosomal particles (Moreno Diaz de la Espina et al., 1980; Goessens, 1984), again indicating an incomplete processing of rRNA precursors and an altered assembling of ribosomal proteins.

The nuclear changes appear to be less evident than nucleolar changes despite the well-known capacity of CDDP to form intrastrand and interstrand crosslinks with nucleic DNA. We suggest that the nuclear changes, such as the focal clearing of the nucleoplasm occurring in CDDP-treated rats, may be the expression of damage to the chromatin of the nucleus, induced by the direct interaction of CDDP with histones of the nucleosomata.

The somatic and nuclear size of DRG neurons decreased significantly after CDDP treatment, suggesting a possible reduction in the rate of protein synthesis. This assumption is supported by the evidence that cellular organelles involved in the synthesis and processing of proteins such as the RER or Golgi apparatus were altered, especially in those rats which underwent the most intense CDDP treatment. On the other hand, we cannot exclude that RER and Golgi apparatus changes may be due to the direct toxic action of CDDP on these organelles. However, the hypothesis of a reduction in the rate of protein synthesis after CDDP treatment does also agree with the reduction in nucleolar activity, as discussed above.

Mitochondrial alterations were very slight and

limited. This finding is somewhat unexpected if one considers that mitochondria contain DNA. A possible explanation may be the protective effect of mitochondrial membranes.

Unlike mitochondria, lysosomes and lipofuscin granules greatly increased in number, and lysosomes showed morphological alterations in CDDP-treated rats. These changes have been related to an increase in intracellular breakdown processes (Müller et al., 1990) due to the direct interaction of CDDP with the macromolecules of cytoplasm organelles.

In conclusion, from this ultrastructural study it can be inferred that CDDP may cause a reduction in the protein synthesis rate in DRG neurons, probably as the result of a decline in nucleolar functions and an alteration of the cytoplasm organelles involved in protein synthesis. This hypothesis would explain why non-mitotic cells with a high rate of protein synthesis, such as the neurons, become pathologically affected by CDDP treatment. Moreover, the lack of an efficient blood/nerve barrier in the DRG (Olson, 1984) explains the involvement of these neurons while the central nervous system neurons are spared.

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