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ORGANOTYPIC CULTURES OF CHICK DORSAL ROOT GANGLIA IN A SEMI-SOLID MEDIUM: A MODEL FOR NEUROTOXICITY TESTING

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Abstract—Organotypic cultures of dorsal root ganglia (DRGs) were isolated from 12-day-old chick embryos and cultured in a semi-solid medium for use as a model for toxicological studies. The mean radial length of neuritic processes growing out from the ganglia, the daily rate of neurite elongation, and the area of neurite outgrowth were used as parameters to evaluate the toxic influence of three drugs (cisplatin, taxol and chlorpromazine) that have neurotoxic side-effects through different modes of action on the peripheral nervous system. A significant, dose-dependent decrease of all parameters of neurite outgrowth was observed in cultures treated with different concentrations of the chemicals tested. We conclude that the culture system described here may be a useful *in vitro* method to test target organ toxicity in the peripheral nervous system.

INTRODUCTION

The use of *in vitro* alternatives to animal models has greatly expanded in experimental toxicology during recent years (Chambers, 1989; Freese *et al.*, 1990; Halks-Miller *et al.*, 1991; Harvey, 1988; Pauwels *et al.*, 1989; Verity *et al.*, 1990). Such alternative models contribute to a better understanding of the mechanisms by which toxic effects are expressed. Moreover, *in vitro* toxicity studies allow the monitoring of toxicity with unprecedented thoroughness and precision at the cellular or tissue level, rather than at the organismal level, and in this way contribute to the improvement of the specificity and sensitivity of animal models (Purchase, 1990). Further development of *in vitro* models will allow a more detailed recognition of cell reactions and interactions following toxic injury, as well as toxicity monitoring in defined target organs.

It may be possible to use cell and organ cultures of dorsal root ganglia (DRGs) to extend our knowledge of mechanisms involved in peripheral nervous system impairment. These cultures have been widely used to study trophic factors which play a role in the survival of sensory neurons *in vitro* (Ebendal, 1989; Letourneau, 1978), the influence of different substrates on neuronal morphogenesis and neurite extension (Bray *et al.*, 1987; Humphries *et al.*, 1988; Letourneau, 1975; Tuttle and Matthew, 1991), and the reactions following peripheral nerve injury (Woolf *et al.*, 1990).

Apart from the study of different common parameters of cell viability and growth, *in vitro* approaches enable the evaluation of indicators specific for nervous tissue, especially with regard to potential to form and extend neuritic processes. The characteristics of neuritic outgrowth have been proved to be factors that are convenient to study and that affect neuronal development, plasticity and survival (Ebendal, 1989; Grothe and Unsicker, 1987; Humphries *et al.*, 1988; Letourneau, 1978; Levi-Montalcini, 1987; Mandys *et al.*, 1991b). They can be used to evaluate nervous tissue damage (Tanii and Hashimoto, 1991).

A semi-solid (soft agar) culture medium has been used successfully to study cellular differentiation (Bjerkvig *et al.*, 1986; Letourneau, 1978), cellular interactions *in vitro* (Bartkova *et al.*, 1987) and organ morphogenesis (Dye and Kollar, 1978). Recently, we described a new culture technique; cultivation of rat spinal cord slices in a soft agar culture medium (Mandys *et al.*, 1991a). This technique is especially suitable for the study of axonal development and regeneration. The soft agar medium we use enables neurons to survive for long periods and to extend neurites, but at the same time prevents cells from migrating from the explants, which would mask the morphology of neurites and change intercellular contacts inside the ganglion. The method has the further

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Abbreviations: CDDP = *cis*-diamminedichloroplatinum (cisplatin); DRG = dorsal root ganglion; DMEM = Dulbecco's modified Eagle's medium; FCS = foetal calf serum; β-NGF = the β-subunit of nerve growth factor; NF = neurofilament.

advantage that we did not have to treat the cultures with substances, such as arabinofuranosylcytosine, which are often used to prevent the proliferation and migration of non-neuronal cells in explant cultures cultured in a liquid medium (Tuttle and Matthew, 1991).

In the present study we adapted the soft agar culture system for the cultivation of chick embryonic DRGs. The aim was to establish morphological characteristics and growth parameters of neurites growing out from the ganglia and to ascertain whether cultivation of DRGs in soft agar culture medium can be a useful model for the study of target organ toxicity in the peripheral nervous system.

MATERIALS AND METHODS

Chemicals. Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS) and a 7.5% solution of $NaHCO_3$ were obtained from GIBCO (Paisley, Scotland), sodium benzylpenicilline was from Centrachemie (The Netherlands), streptomycin sulfate and cisplatin (*cis*-diamminedichloroplatinum, CDDP) were purchased from Pharmachemie (The Netherlands). Agar (DIFCO Agar Noble) was purchased from DIFCO Laboratories (Detroit, IL, USA). β-NGF (the β-subunit of nerve growth factor) was from Boehringer (Mannheim, Germany). Taxol was purchased from Bristol-Myers Squibb, Woerden (The Netherlands) and chlorpromazine (Largactil) from Rhône-Poulenc (France). Sterile plastic ware was obtained from Nunc (Roskilde, Denmark).

Culture experiments. A two-layer culture system, as previously described (Bartkova *et al.*, 1982; Mandys *et al.*, 1991a), was used. The lower agar medium layer, consisting of 1.5 ml culture medium supplemented with 0.5% agar, was pipetted into 35-mm petri dishes. Freshly isolated DRGs from 12-day-old chick embryos (HH stage 37–38) were placed on top of this layer and then the upper agar medium layer, consisting of 1 ml culture medium with 0.5 or 0.25% agar, was added in such a way that the DRGs were fully embedded in agar. At least eight ganglia were used for each experimental condition in 25 independent experiments. The culture medium consisted of DMEM supplemented with 10% FCS, 100 U benzylpenicilline/ml and 100 μg streptomycin sulfate/ml. A low amount of β-NGF (2–3 ng/ml) was necessary for neurite extension. Based on previous experiments, this concentration is at the lower end of the linear part of the β-NGF/neurofilament (NF) dose–response curve (data not shown) and appears to be optimal to study the influence of different growth-promoting substances on intact dorsal root ganglia. The pH of culture medium was adjusted to 7.2–7.4 with a 7.5% solution of $NaHCO_3$. The petri dishes were kept in a humidified atmosphere of 6.5% CO_2 in air at 37°C.

Drug treatment. Different amounts of cisplatin, taxol or chlorpromazine were added to the culture medium in both the lower and upper layer. The final concentration in the culture medium ranged from 0.33 to 66 μM (0.1–20 μg/ml) for cisplatin, from 1.4 to 45 μM (0.5–16 μg/ml) for chlorpromazine, and from 11.7 nM to 23.4 μM (0.01–20 μg/ml) for taxol. In all of these experiments, 0.25% agar medium was used for the upper layer.

Morphology and morphometry of the cultures. The cultures were examined over 4 consecutive days using an Olympus IM Microscope or an OPTON Axiovert 35 Microscope. Morphological changes of neuritic processes growing out from the DRGs were evaluated using phase contrast and dark field illumination. Several approaches were used in two independent laboratories to determine the changes of neuritic outgrowth.

(1) Direct measurement of the radial length of neurites growing out from the ganglia in native cultures was performed using an Olympus OSM Micrometer. The processes were measured from the edge of the ganglion to the growth tips at at least three different sites of each ganglion. One to three processes were measured in one observation, depending on the density of the outgrowth. Dichotomies of neurites could not be taken into account, because of the three-dimensional arrangement of the processes (seven experiments).

(2) Pictures of cultured DRGs were made either by tracing the contours on a plastic sheet directly from the screen of a video monitor or by taking photographs. These pictures or photographs were then used for morphometric measurement of neurite outgrowth using a Mini-Mop morphometric device (Kontron, Germany) and a graphic tablet. Two parameters of neuritic outgrowth were evaluated: the radial length of the processes and the area of outgrowth. These values were used to calculate the daily rate of neurite elongation (μm/day) and the area index of neuritic outgrowth, defined as area of outgrowth divided by area of the ganglion to correct for the size of the ganglion (16 experiments).

(3) In two experiments we were able to compare the measurement of the area as described above with the results from a computerized morphometric approach. Under dark-field illumination the image of DRG plus outgrowth was digitized and manipulated with an image processing system consisting of shading correction, background subtraction and contrast stretch. Next, the image of the DRG under normal illumination was digitized and subtracted from the total picture, leaving only the pixels representing outgrowth on screen. The number of pixels can then be used for further calculations (under the conditions used 5 pixels = 1 μm^2). All measurements were performed 'blind' (i.e. the investigator was unaware of the treatment of the samples).

Statistical analysis. To test the significance of changes in neurite length, growth rate and area index as a function of time and dose of neurotoxin, a multiple analysis of variance was used (MANOVA) as implemented in the SPSS/PC + statistical package. The significance of dose effects was occasionally

tested at one time point by using an ANOVA. For the significance of single doses of drugs on growth parameters Bonferroni's t-test was used following an ANOVA.

RESULTS

Morphology and basic growth characteristics of neuritic processes

Neurites growing out from DRGs appeared within the first 12 hr *in vitro*. They grew very regularly with a radial arrangement, usually symmetrically around the whole perimeter of the ganglia (Plate 1a). The processes revealed conspicuous branching and, under higher magnification, growth cones could be observed. The radial length of the processes, as well as the daily rate of their elongation, were dependent on the concentration of agar in the upper layer. In 0.5% agar medium the processes grew very slowly, their daily growth rate being about 52 μm during the first 5 days of cultivation. The mean radial length of these processes was 285 μm after 5 days in culture (Fig. 1). In 0.25% agar medium the mean daily growth rate of the neuritic processes ranged in different experiments from 125 to 210 μm within the first 5 days in culture, depending on the concentration of β-NGF in culture medium (2 or 3 ng/ml). The mean radial length of these processes ranged in accordance with the growth rate from 630 to 950 μm after 120 hr *in vitro* (Fig. 1).

The effect of chemical agents on the morphology of neurites

Cisplatin. The outgrowth of neuritic processes was not influenced by low doses of cisplatin (up to 6.6 μM in both agar layers). Morphologically, the first changes could be observed at 26.4 μM cisplatin. The processes still grew out around almost the whole perimeter of the ganglia, but their shape was slightly irregular. Some of the processes were thicker and revealed irregular branching, or distortion (Plate 1b). Severe morphological damage, characterized by prominent irregularities of the shape of neuritic processes with distortions and only occasional branching were observed at 52.8 μM cisplatin. The neurites grew out only from a few parts of the ganglion (Plate 1c). Some processes could still be observed even at a relatively high concentration (66.6 μM; Plate 1d).

Chlorpromazine. Morphological changes of neurites growing out from the ganglia treated with chlorpromazine occurred at low concentrations of the drug (5.6 μM) and were characterized by a large variation in the length of the processes (Plate 2a). A significant reduction in the number of neurites growing out from the ganglia as well as in their length and branching could be observed at a higher concentration of chlorpromazine (22.4 μM). Disintegration of processes was seen in some of these cultures after 96 hr of cultivation. Neuritic processes were observed occasionally at a concentration of 33.6 μM. The majority of these processes underwent disintegration within 48 hr of cultivation (Plate 2b).

Taxol. Ganglia treated with taxol displayed different morphological responses from those described for cisplatin and chlorpromazine. Taxol reduced predominantly the length of the processes, which grew out around the whole perimeter of the ganglia at 11.7 and 58 nM taxol (Plate 2c). At a higher concentration of taxol (117 nM) the neurites were very short, but they were still growing out around almost the whole perimeter of the ganglia. Branching of the processes was reduced (Plate 2d).

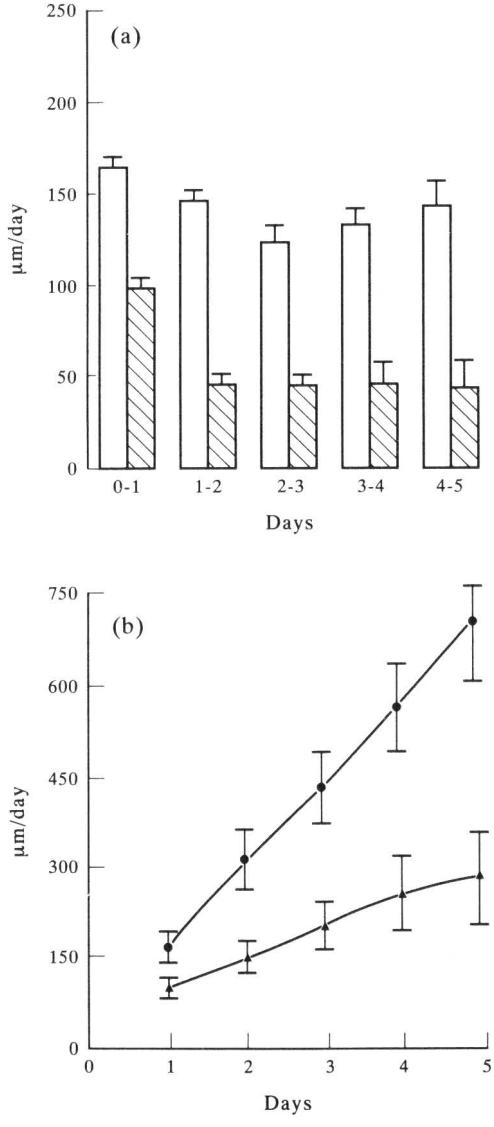

Fig. 1. (a) Comparison of the daily rates of neurite elongation in different agar concentrations in the upper layer of the culture medium: ■, 0.25% agar; ▨, 0.5% agar. Error bars indicate the SEM. (b) Influence of different concentrations of agar in the upper layer on the mean radial length of the neurites growing out from the ganglia: ●, 0.25% agar; ▲, 0.5% agar. Error bars indicate the SD.

The effect of drugs on the growth parameters of neurites

Statistical analysis (MANOVA) showed that all three drugs had a significant inhibitory effect ($P < 0.001$) on outgrowth, as reflected by all three parameters used: neurite length, rate of elongation, and area index. At each time point, even at the earliest (24 hr *in vitro*), the effect of the drugs was significant (ANOVA, $P < 0.001$). In almost all cases on any one day, the values at different concentrations differed significantly both from the control and from each other (Bonferroni t-test). We indicate below when this was not the case. The results obtained with image processing correlated in a highly significant way with the data obtained with the digitization method. However, because the computerized method was not available for all experiments we used only the data obtained by direct measurement of neurite length or by digitization of outgrowth from pictures.

Cisplatin. The changes in growth parameters of neurites from ganglia treated with cisplatin were clearly dose dependent (Fig. 2). The rates of neuritic outgrowth were consistently lower over the whole period of cultivation. Accordingly, the mean radial length of the processes was smaller. The reduction of neuritic processes is reflected by the regular decrease of the area index of outgrowth. All observations were significantly different from controls and each other at each time point.

Chlorpromazine. The lowest dose of chlorpromazine studied (5.6 μM) did not change the rate of neuritic outgrowth nor the mean radial length of the processes significantly within the first 48 hr of cultivation. However, from 72 hr onwards, both the rate of neurite elongation and the mean radial length were significantly reduced at this concentration. Thus, the effect of chlorpromazine, especially at the lower two doses, tends to appear only at a later stage of development *in vitro*. In contrast, at the highest dose studied (33.6 μM), outgrowth of a small number of neurites could be measured only within the first 24 hr *in vitro*, because after that time outgrowth stopped completely (Fig. 3).

Taxol. Growth parameters of neurites in cultures treated with taxol were reduced in accordance with the morphological effects. In contrast to the cultures treated with chlorpromazine, the rate of daily neuritic outgrowth in the cultures treated with high concentrations of taxol (58 or 117 nM) increased after a long period of cultivation (72–96 hr; Fig. 4). The time–effect curves for the mean radial length of the processes showed no significant differences between the two highest concentrations. However, the other curves were highly significantly dose dependent, and significant differences between doses were also observed (at all doses) for the other growth parameters measured.

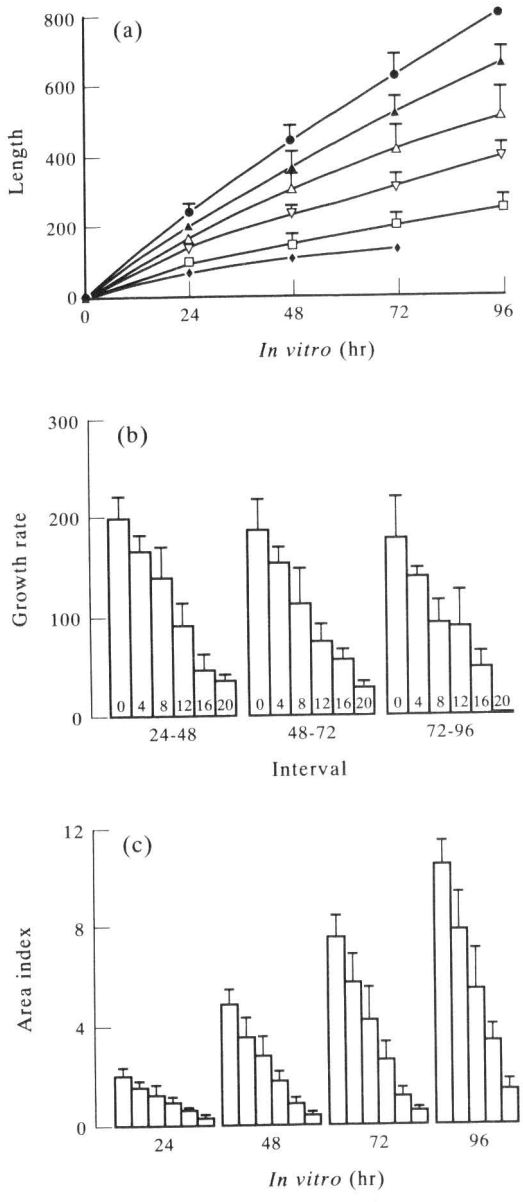

Fig. 2. Effect of different concentrations of cisplatin on the parameters of neuritic outgrowth. (a) Mean radial length of the neurites is given in μm. Doses used: 0 (●), 4 (▲), 8 (△), 12 (▽), 16 (□) and 20 (◆) mg/litre (0, 13, 26, 40, 53 and 67 μM, respectively). (b) Daily rate of neurite elongation calculated as the increase in length of neurites during a given interval (x-axis, interval in hours *in vitro*) and expressed as μm/day. (c) Area index (ratio between surface of outgrowth and surface of ganglion). The error bars indicate the SD.

DISCUSSION

The quantitative and qualitative characteristics of neurite outgrowth reflect the functional potency of a culture system. In the system used in the present study it has been demonstrated that the quality of the substrate as well as the composition of the culture medium is very important for the optimal development of the cultures (Baron-Van Evercooren *et al.*, 1982; Coughlin and Kessler, 1982; Ebendal, 1989;

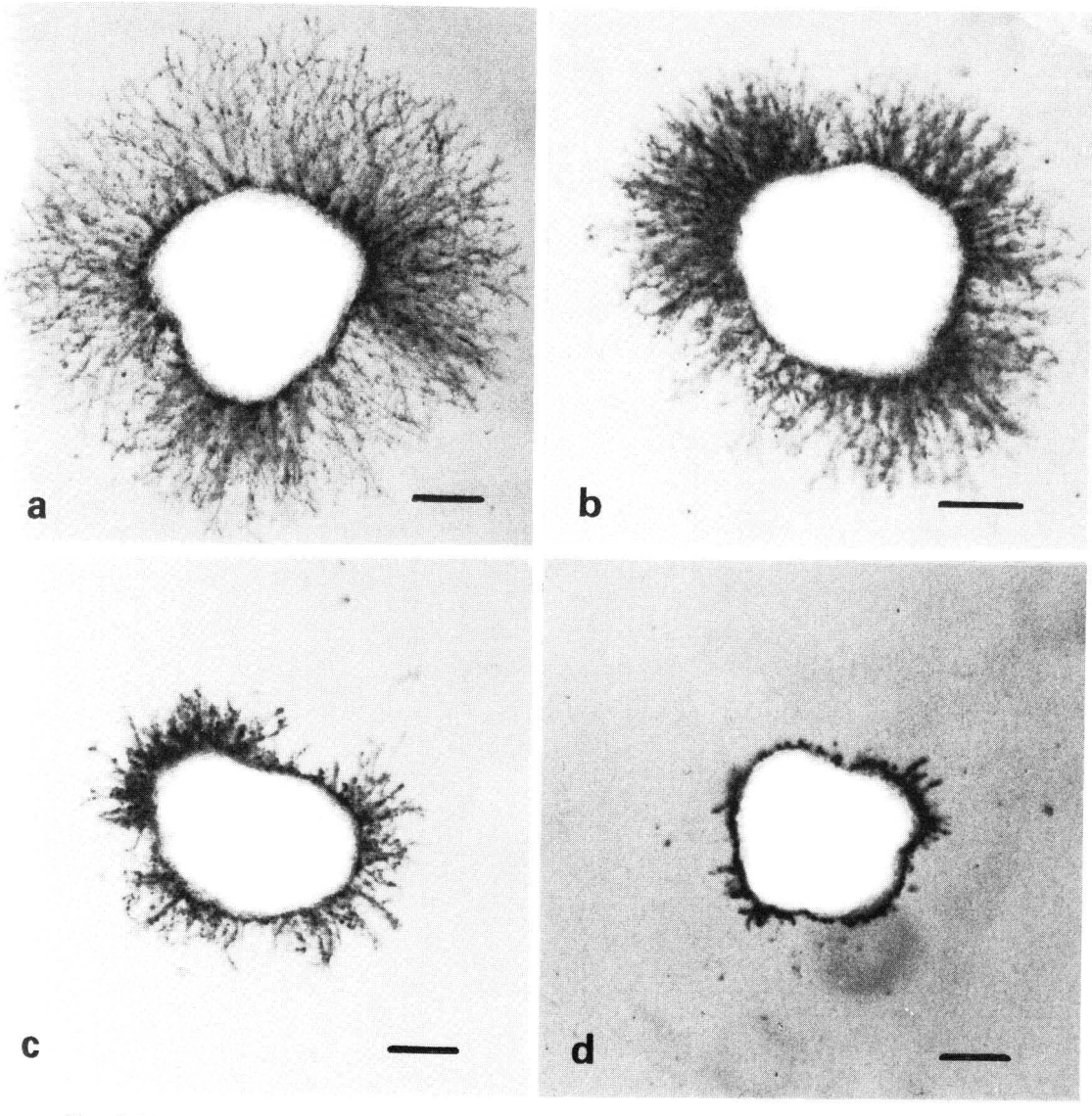

Plate 1. (a) Outgrowth of neurites from a ganglion in 0.25% agar medium. (b) Neurites growing out from a ganglion treated with 26.4 μM cisplatin. (c) Neuritic outgrowth after treatment with 52.8 μM cisplatin. (d) Very few, short processes growing out from a ganglion treated with 66.6 μM cisplatin. All ganglia were photographed at 48 hr *in vitro*. Bars represent 200 μm.

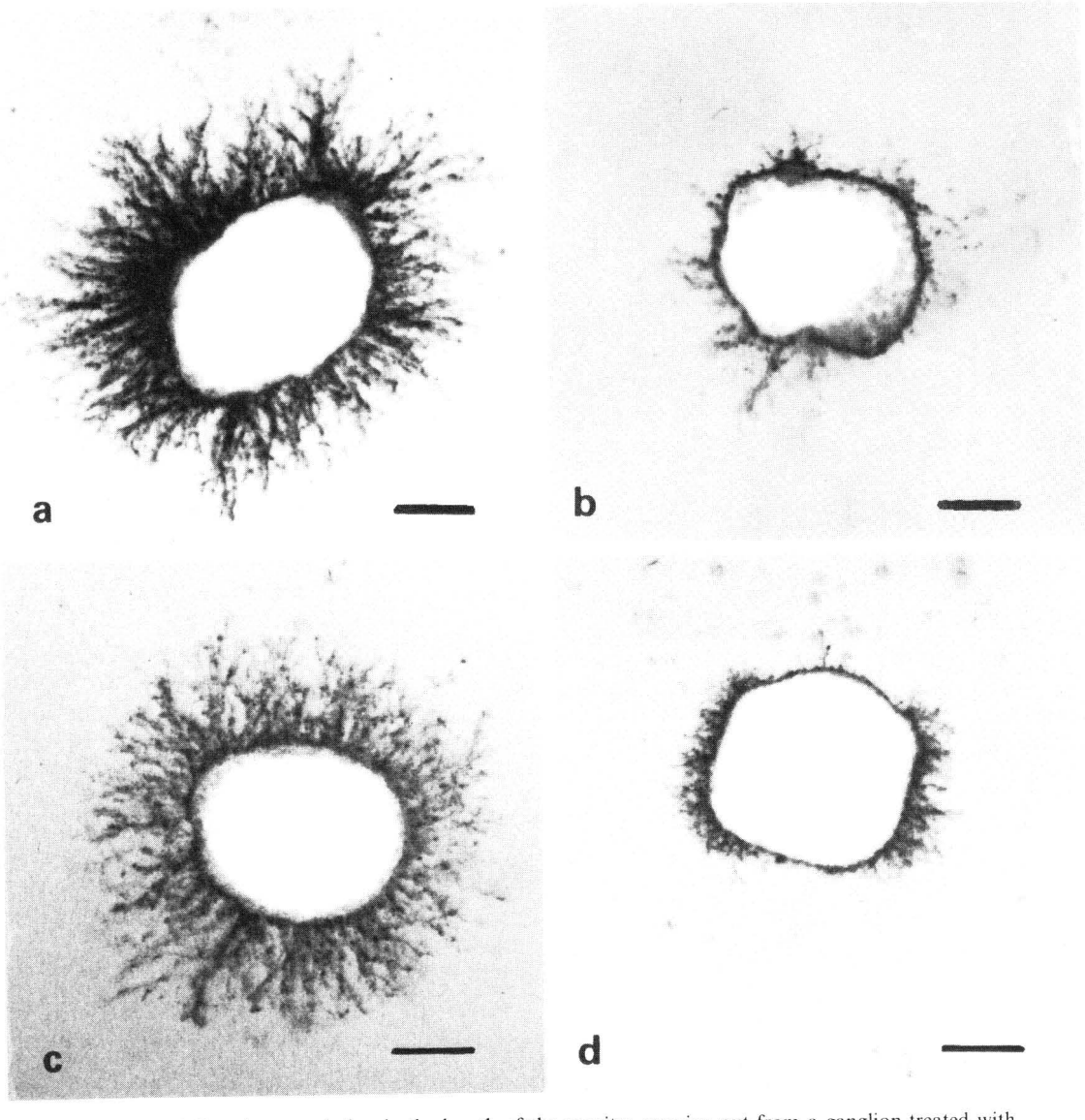

Plate 2. (a) Prominent variation in the length of the neurites growing out from a ganglion treated with 5.6 μM chlorpromazine. (b) Disintegration of neurites in a culture treated with 33.6 μM chlorpromazine. (c) Regular outgrowth of shorter neurites around a ganglion treated with 11.7 nM taxol. (d) Relatively high number of very short neurites growing out from a ganglion treated with 117 nM taxol. All ganglia were photographed at 48 hr *in vitro*. Bars represent 200 μm.

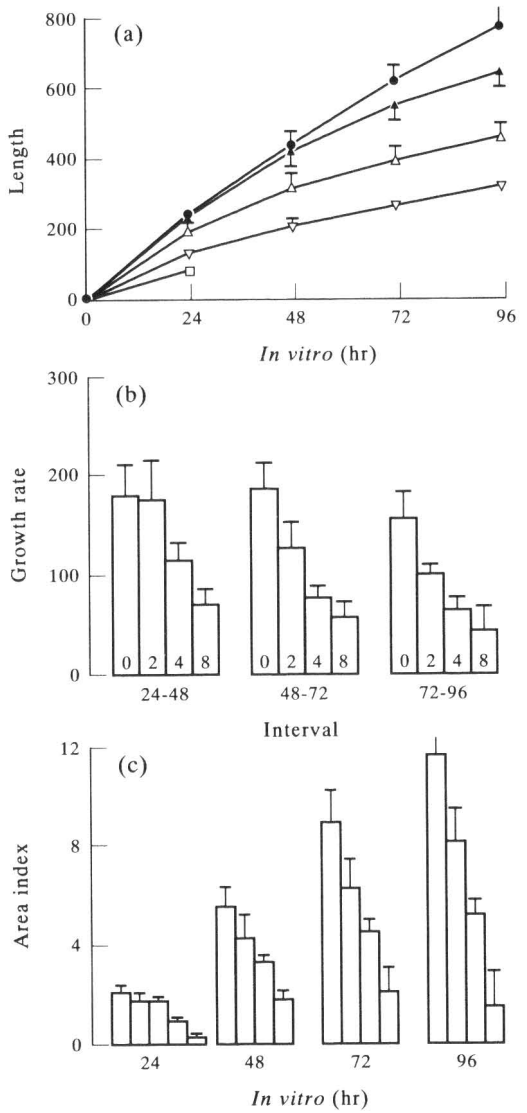

Fig. 3. Effect of different concentrations of chlorpromazine on the parameters of neuritic outgrowth. (a) Mean radial length of the neurites in μm after doses of 0 (●), 2 (▲), 4 (△), 8 (▽) or 12 (□) mg/litre (0, 6, 11, 22 and 34 μM, respectively). (b) Daily rate of neurite elongation (μm/day). (c) Area index (ratio between surface of outgrowth and surface of ganglion). The measurements were performed on a graphic tablet. Error bars indicate the SD.

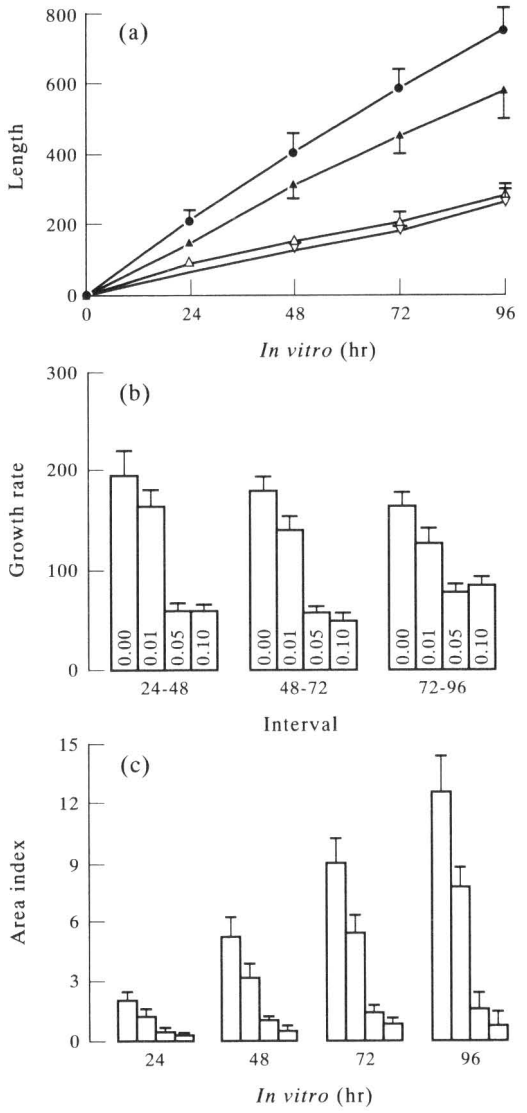

Fig. 4. Effect of different concentrations of taxol on the parameters of neuritic outgrowth. (a) Mean radial length of the neurites in μm after doses of 0 (●), 0.01 (▲), 0.05 (△) or 0.1 (▽) mg/litre (0, 12, 15 or 117 nM, respectively). (b) Daily rate of neurite elongation (μm/day). (c) Area index (ratio between surface of outgrowth and surface of ganglion). Error bars indicate the SD.

Humphries *et al.*, 1988; Letourneau, 1975; Levi-Montalcini, 1987).

The concentration of agar in the culture medium plays an important role in obtaining optimal culture results (Bartkova *et al.*, 1982; Mandys *et al.*, 1991a). For dorsal root ganglia, we found a concentration of 0.25% agar in the upper medium layer was optimal. Lower concentrations do not form a strong enough matrix, whereas higher concentrations slow down outgrowth too much. Using this concentration we obtained an extensive and highly regular outgrowth of neuritic processes, which appeared to be very reproducible within and between experiments. This enabled a detailed morphological evaluation of the processes and precise measurement of several outgrowth parameters. The position of the ganglia inside the upper agar medium layers did not play any role in the development of neurites. Care had to be taken only that the ganglia were fully embedded in the medium.

The effect of all three chemicals tested on neuritic outgrowth was unambiguously dose dependent. Nevertheless, we found marked differences in the morphology as well as in the growth parameters of the neurites growing out from the ganglia exposed to the different chemicals. The growth parameters of the

neurites were inversely correlated with the dose of all three chemicals.

Cisplatin displayed the lowest neurotoxicity of the three drugs used. A change in growth rates and a decrease in the number of neuritic processes growing out from the ganglia could be observed only at relatively high concentrations of CDDP (more than 13.2 μM). The observation that, even at 66.6 μM, outgrowth of neurites was not completely blocked, indicates that the neurons are relatively less sensitive to CDDP than are some other cell types, such as fibroblasts (Cervinka and Drobnik, 1984) and Schwann cells: even at 1 μg cisplatin/ml (3.3 μM) 50% of cultured Schwann cells die (Sodaar *et al.*, 1989). Another explanation of the relatively low sensitivity of neurons to cisplatin *in vitro* can be based on the finding that non-neuronal cells *in vivo* accumulate much more cisplatin than neurons (Terheggen *et al.*, 1989). The toxic effect of cisplatin is influenced by binding of the drug to proteins, especially under *in vivo* conditions (Daley-Yates and McBrien, 1984). Protein binding could be considered a factor influencing toxicity in cell and tissue cultures, because the culture medium usually contains 5–10% of animal serum or other proteins. Short-term treatment of cell cultures with cisplatin (1–3 hr) does not change the activity of the drug (Akaboshi *et al.*, 1992). A time-dependent decrease in cell survival was observed in the experiments of Akaboshi *et al.* (1992). It is reasonable to assume that the activity of cisplatin decreases during cultivation, as it does under *in vivo* conditions. A possible influence of protein present in the culture medium could easily be studied in the system described here.

The toxic action of chlorpromazine seems to be based predominantly on the formation of free radicals and on interference with different metabolic processes within the cells, for instance the blockade of Na^+ and Ca^{2+} channels (Messiha, 1991; Ogata *et al.*, 1990). The toxic effect of chlorpromazine in our cultures was in agreement with the idea that neurons are directly influenced by this drug and is supported by the finding of extensive disintegration of the neurons in later phases of cultivation. The neurotoxic dose range of chlorpromazine in our experiments is comparable with that obtained in embryotoxicity and genotoxicity studies (Lialiaris *et al.*, 1992; Peterka *et al.*, 1992). On the basis of the results of embryotoxicity tests we can conclude that the concentration of the drug used *in vitro* corresponds to 1.0–10.0 mg/kg body weight in animal studies. A similar concentration of chlorpromazine was used to study the neurotoxicity of the drug *in vivo* (Messiha, 1991).

Our observation that taxol influenced predominantly the prolongation of the neurites in our culture system is in agreement with the previous finding that taxol induces unusual arrays of microtubules and in that way may inhibit neuritic outgrowth (Letourneau and Ressler, 1984; Masurovsky *et al.*, 1981 and 1983). Taxol was about 200 times more toxic (on a molar basis) than both of the other chemicals tested, and our results were similar to the findings in dissociated DRGs (Letourneau and Ressler, 1984; Letourneau *et al.*, 1986). The increase in the rate of neurite elongation in taxol-treated cultures in later phases of cultivation points at a regenerative potency, which was also observed in the neurons of dissociated dorsal root ganglia after removal of the drug from culture medium (Letourneau and Ressler, 1984).

The neurotoxicity of a drug *in vivo* is determined by, amongst other factors, its total body clearance. The disappearance of the drug from plasma is a multiphasic process, in which binding to serum proteins and other cellular components plays a substantial role (Wiernik *et al.*, 1987). Previous studies have shown a large inter-patient variability in the pharmacokinetics as well as in the toxic effects of taxol (Brown *et al.*, 1991).

The use of a low dose of NGF in our experiments deserves separate attention, because NGF has been shown to prevent a neuropathy in mice treated with cisplatin (Apfel *et al.*, 1991). NGF was a prerequisite for neurite outgrowth in our cultures, but the dose used (2 ng/ml) produced only 5–10% of the maximal outgrowth that was obtained by higher doses of NGF (30 ng/ml). Studies are currently in progress to see whether high NGF acts as neuroprotective agent *in vitro* as well. Since medium containing serum and NGF has been used in studies on changes occurring in DRGs exposed to taxol (Masurovsky *et al.*, 1983), our findings can be compared with the results of these studies.

Organotypic cultures represent a useful approach to study organ-specific toxic mechanisms. These culture systems preserve the internal organization of the tissue and keep intercellular contacts (Gähwiler, 1988; Syversen, 1991). From this point of view, our culture system seems to fulfil the criteria for an alternative toxicity test (Harvey, 1988; Van den Heuvel and Fiedler, 1990). It is simple, rapid, sensitive, reliable and precise enough for target organ toxicity studies within the peripheral nervous system.

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