SENSITIVITY OF SEVERAL CELL SYSTEMS TO ACRYLAMIDE

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SUMMARY

Chick spinal ganglia, chick muscle cells combined with mouse spinal cord explants, C1300 neuroblastoma cells, Chinese hamster ovary cells and newborn rat cerebral cells were exposed to various concentrations of acrylamide in culture. Four morphological and one electrophysiological parameters were applied in order to score toxic effects. It appeared that the neurite formation of rat cerebral neurons was the most sensitive criterion showing an effect at 10^{-7} M acrylamide.

INTRODUCTION

The use of cultured cells for relatively inexpensive and rapid screening of mutagenic effects of chemicals is widely adopted and justified by the scarcity of toxicological data about the large amount of old and newly synthesized compounds. The methods used are relatively simple when compared to those employed for screening for neurotoxicity, due to the diversity of functional and anatomical units in the nervous system which may be affected. For neurotoxicity screening it may, therefore, be necessary to select the most sensitive criterion in the most suitable cell culture system. This is cumbersome and would require the development of a great number of standardized cell culture systems. On the other hand the prospects might be less grim when it could be shown that a culture system can be found which is suitable for the screening of neurotoxic agents which cause similar neurotoxic effects.

The first results of such an approach, whereby a culture system most sensitive to acrylamide was selected from six different types of culture, is presented here. In the future the same screening method will be used for compounds which are known to cause similar effects.

MATERIALS AND METHODS

Tissue culture

The tissue culture methods have been summarized in table 1. More details of the methods used can be found in the references cited (1, 2, 3).

Scoring methods

Four morphological parameters were applied to assess the cytotoxicity of acrylamide for chick muscle cells; i.e. the cross striation, the thickness, the number of muscle cells and the absence of vacuoles (4). For each of the four parameters the condition of culture was rated according to a five point scale from 0-4, with 4 for the best condition. The cultures were exposed to acrylamide during 18 days and they were scored three times. The presence of end plates in mixed cultures of muscle cells and spinal cord explants was investigated with conventional intracellular electrophysiological techniques (2). Records of intracellular electrical activity of 2 min duration obtained from 20 randomly selected muscle cells per culture dish in the vicinity of a mouse spinal cord explant were inspected for the presence of end plate potentials and their frequency of firing was established. In plating efficiency tests cultures were exposed to acrylamide during one week. Cell colonies were counted after incubation of the cultures with 5% methylene blue in Tyrode's solution. The growth cones of the processes from chick spinal ganglia were counted in the living culture on the thermostated stage of an inverted microscope with phase- contrast optics. Cerebral cells obtained by trypsin dissociation from newborn rat whole cerebra (3) were counted in 10 randomly chosen fields (diameter 800 μm). Flat cells were excluded. In the same fields also the number of cells that had formed neurite-like extensions longer than 25 μm was established. The visibility of neurites was enhanced by impregnation of the cells with methylene blue.

RESULTS

The results of all experiments have been summarized in table II. For comparison, the results of the study of Sharma and Obersteiner (5) have been included. To our knowledge this is the only other study of the neurotoxic effects of acrylamide using cultured neurons.

Spinal ganglia

Isolated spinal ganglia in culture formed a corona of neurites and glia cells after several days in culture. Most neurites exhibited a specialized ending generally called a growth cone. Growth cones were only counted if the ganglion showed a symmetrical outgrowth. The exceptional ganglion showing an outgrowth that deviated more than 10% from the average outgrowth in the whole group was discarded. In this way approximately 10% of the ganglia that had been damaged during dissection were excluded. Notwithstanding the symmetrical appearance of the corona it appeared misleading to count growth cones only in a restricted area and then calculate their total number by multiplication with the total area of corona, this practice introduced large errors. The radius of the corona appeared independent of exposure to acrylamide (10^{-6}-10^{-4} M) during three days. The number of growth cones after three days in culture, however, was 168 $\pm$ 31 and 92 $\pm$ 12 (mean $\pm$ SEM), in control and acrylamide exposed (10^{-4} M) ganglia, respectively. Lower concentrations had no effect.

Muscle cells

In order to make sure that any effect of acrylamide on the formation of end plates that might be detected in mixed cultures of muscle cells and spinal cord explants was not due to a direct action on the muscle cells, the cytotoxicity of this compound for muscle cells had to be established. Compared with non-exposed cultures only a very small decrease in the number of muscle cells was detected in cultures that had been continuously exposed to the highest concentration of acrylamide (10^{-4} M).

Mixed cultures

Chick muscle cells become multiply innervated within two days after the addition of spinal cord explants. The cultures were exposed to acrylamide (10^{-6}-10^{-4} M) during 3, 5 and 16 days after the addition of the spinal cord explants. No difference was observed in the number of innervated muscle cells or the frequency of end plate potentials in exposed and non-exposed cultures. However, since the available electrophysiological techniques did not allow estimation of the number of end plates on individual muscle cells, a reduction in multiplicity of innervation may have remained undetected.

Plating efficiency

This technique has been widely adopted to establish the cytotoxicity of compounds. In the present experiments cells of neuronal and non-neuronal origin were studied, the C1300 mouse neuroblastoma and the Chinese hamster ovary (CHO) cell line, respectively. After continuous exposure during one week to the highest concentration of acrylamide (10^{-5} M) the plating efficiency of C1300 and CHO cells was decreased by 16% and 11%, respectively, differences which were not statistically significant from the control values.

Cerebral cells

Freshly trypsin-dissociated cerebral cells started to form neurite-like extensions within 2 hours after plating on poly-l-lysine. After 16 hours in culture a great number of cells with neurites of up to 600 um were present, probably these were neurons. Some cultures were regularly inspected up to 48 hours. At that time deterioration of the neurons became apparent, resulting in loss of neurites. Flat cells present in the same culture did not seem to be affected and survived for at least 2 weeks. Similar observations have been reported bu Hauser and Bernasconi (3). In order to use the most vigorous

phase of neurite formation the acrylamide experiments were carried out during the first 16 hours in culture. During this period the cultures were continuously exposed to acrylamide (10^{-7}, 10^{-5} M). Figure 1 shows the results of these experiments. A dose related and statistically significant reduction of the number of neurons with neurites was observed.

DISCUSSION

Newborn rat cerebral cells appear to be very sensitive to acrylamide. Of the other cell types investigated, only neurons of spinal ganglia were affected, albeit at a very high concentration (10^{-4} M). This figure is well in agreement with the concentrations reported by Sharma and Obersteiner (5). The difference in sensitivity of these two culture systems might be ascribed to a difference in vulnerability between cells cultured as single cells and those grown in histotypic explants. Nelson and Bergey (6), for instance, reported that a blockade of impulse transmission caused by tetrodotoxin caused degeneration of single dissociated spinal cord neurons. In contrast, such a blockade caused by xylocaine did not affect the same type of neurons in spinal cord explants (7). Moreover, the cerebral cells in the present study were grown deliberately in such marginal conditions that even without intoxication long survival was impossible (3).

In vivo, toxic amounts of acrylamide first damage long large-diameter sensory axons, sometimes followed by damage to motor axons (8). Such damage might be the result of the disruption of slow axonal transport of protein

by acrylamide, as demonstrated in cats by Pleasure et al. (9). Spencer and Schaumburg (10) suggested that in intoxications leading to a dying-back process enzymes distributed throughout the axon and probably involved in axonal transport are affected and that de novo supply of enzymes in long axons from the perikaryon is insufficient under such conditions. In this respect it is relevant that neuron specific enolase, an enzyme possibly involved in the energy supply for axonal transport, can be considerably inhibited in vivo and in vitro by acrylamide (11). At this moment it is not clear whether and how these findings are related to the inhibited formation of neurites reported here. Schotman and co-workers (12) have reported a reduction of the incorporation of labeled aminoacids in neuronal tissue by acrylamide intoxication. If protein synthesis is also reduced in cultured neurons, formation of neurites will certainly be affected. In addition, interruption of axonal transport in neurites that have started to develop can be expected to cause an extra delay in outgrowth. It will be worthwhile to test such a hypothesis in the near future.

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TABLE I
TISSUE CULTURE METHODS

cell type	embryonic age	culture medium	additives	culture surface	cell number per sq.cm
chick spinal ganglia	11 days	modified Eagle's MEM (2)	5-10 BU NGF 10% HS (h-i) 10% FBS	reconstituted rat collagen	
chick leg muscle cells	11 days	modified Eagle's MEM (2)	15% HS 5% EE	reconstituted rat collagen	5.10^4
chick leg muscle cells + mouse spinal cord explants	11 days 13 days	modified Eagle's MEM (2)	15% HS 5% EE	reconstituted rat collagen	5.10^4
C1300 neuroblastoma and CHO cells		modified L15 (1)	5% HS (h-i) 5% FBS	tissue culture dish (Greiner)	35
rat cerebral cells	newborn	modified L-15 (3)	3% HS (h-i)	poly-l-lysine	3.10^4

HS = horse serum (Flow, Scotland)
HS (h-i) = heat inactivated horse serum (56°, 30 min)
FBS = foetal bovine serum (Sera-lab, U.K.)
NGF = nerve growth factor (Collaborative Research, USA)
MEM = minimal essential medium (Gibco, USA)
EE = freshly prepared chick embryo extract

TABLE II

CULTURE SYSTEMS INVESTIGATED

culture system	parameter	effect	conc. causing an effect (M)	conc. used (M)
chick spinal ganglia	number of growth cones	+	10^{-4}	10^{-6}-10^{-4}
chick muscle cells	cytotoxicity index (4)	$\pm$	10^{-4}	10^{-4},10^{-5}
mouse spinal cord explant. + chick muscle cells	frequency of innervation	-		10^{-6}-10^{-4}
C1300 neuro-blastoma	plating efficiency	-		10^{-9}-10^{-5}
CHO cells	plating efficiency	-		10^{-8}-10^{-5}
rat cerebral cells	neurite formation	+	10^{-7}	10^{-7},10^{-5}
Sharma and Obersteiner (5)			conc. causing half max. effect	
chick spinal ganglia	cytotoxicity index (5)			
	nerve fibres	+	$2.1\ 10^{-4}$	10^{-6}-10^{-2}
	neuroglia	+	$3.8\ 10^{-4}$	

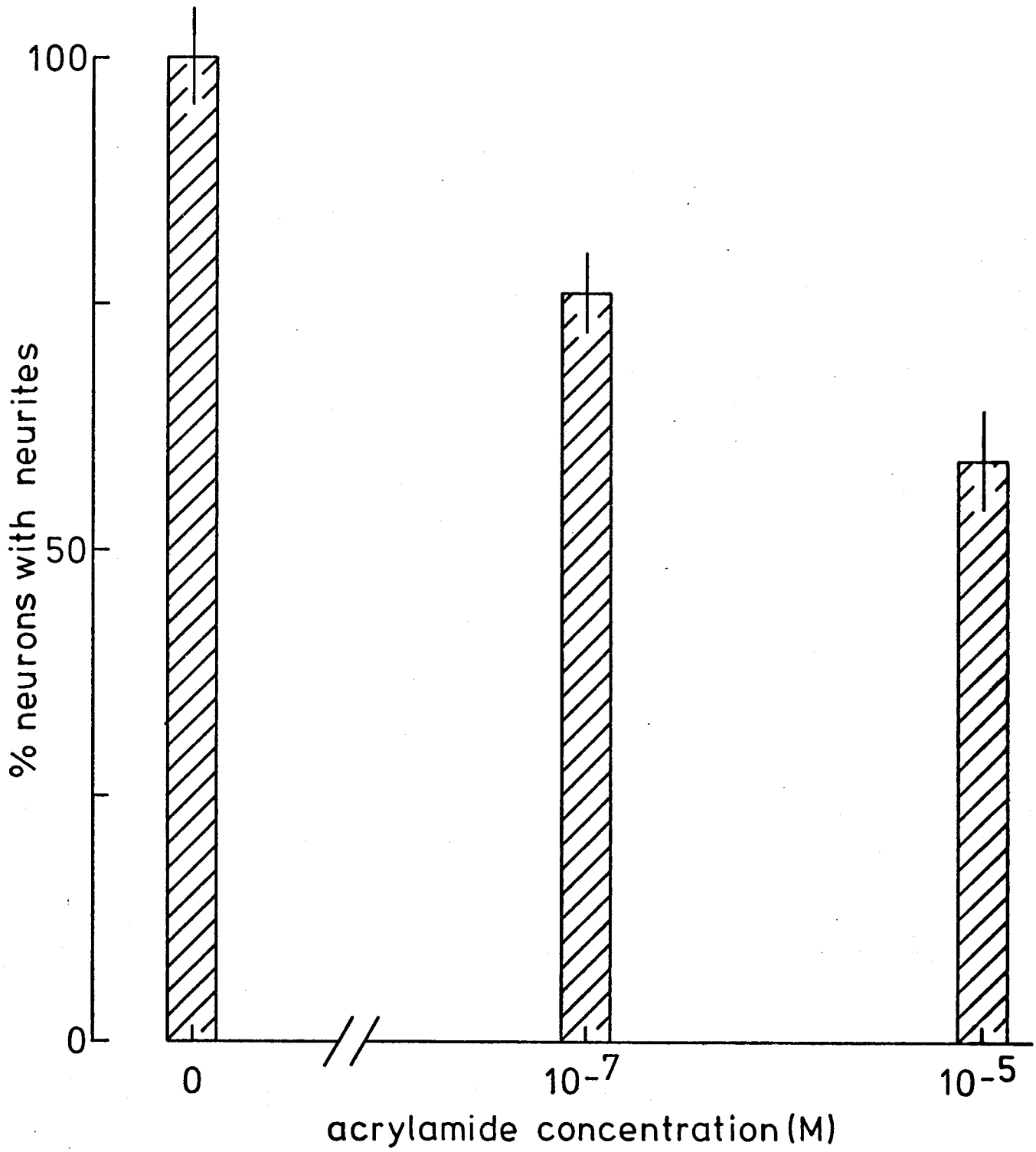

Fig. 1. Reduction by acrylamide of the number of neurons with neurites. The combined results of 3 different experiments each of 18 cultures are presented. The counts of individual cultures were expressed as a percentage of the average number (= 100%) in the control group of each of the 3 experiments. The means ± S.E.M. are presented.

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