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VINBLASTINE-INDUCED AUTOPHAGOCYTOSIS IN EHRLICH ASCITES CELLS

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ABSTRACT

Quantitative electron microscopic analysis was made of vinblastine-induced autophagocytosis in cultured Ehrlich ascites cells in order to test the suitability of the cells as a model in studies of the mechanism of autophagocytosis. The cells were incubated for 2 h in a medium containing 10^{-4} M vinblastine. Samples for morphometric analysis were collected after 3 min, 30 min, 1 h and 2 h incubations. The volume density of autophagosomes had already increased significantly (approximately 15-fold; $p < 0.05$) within 3 min during vinblastine incubation when compared with the controls. This difference between vinblastine-incubated and control cells was maintained throughout the remaining experimental period. The increase in the volume density of autophagosomes was due to the significant increase in their number. We suggest that the experimental model presented offers a useful tool for further studies on induced autophagocytosis.

INTRODUCTION

Various experimental model systems have been used in studies on cellular autophagocytosis (Arstila and Trump, 1968; Hirsimäki et al., 1976; Mortimore and Schworer, 1977) in order to increase the number of autophagic vacuoles (AV) in the cytoplasm, since the turnover of AV's is a rapid process (Mortimore and Schworer, 1977; Pfeifer, 1978) which occurs rather infrequently in normal cells (Hirsimäki and Pilström, 1982). The most suitable model system may be cell culture because the drugs apparently have an effect on the transport and secretion of hormones and related compounds in in vivo experiments. The microtubule inhibitor vinblastine is a

powerful inducer of autophagocytosis in rat (Arstila et al., 1974) and mouse liver (Hirsimäki and Pilström, 1982). The aim of the present study was to estimate morphometrically both the volume density and the number of autophagic vacuoles in Ehrlich ascites cells incubated in a medium containing vinblastine in order to develop a simple model for further studies on induced autophagocytosis.

MATERIALS AND METHODS

Cell harvesting and experimental conditions

Cells were grown in the peritoneal cavities of NMRI mice. Six to ten days after the inoculation, cells were collected aseptically with plastic syringes. They were then separated from the ascites fluid by centrifugation (300 g x 3 min), washed twice with modified Krebs-Ringer phosphate buffer (King et al., 1954), supplemented with glucose (1 mg/ml) and diluted with the same buffer to a concentration of 10^7 cells/ml. Cell suspension was divided into 15 ml aliquots and incubated in Erlenmeyer flasks in a water-bath shaker at +37°C in all experiments. Vinblastine sulphate (Velbe^R, E. Lilly Co.) was used at a concentration of 10^{-4} M. Samples for morphometric electron microscopic analysis were collected after 3 min, 30 min, 1 h and 2 h incubation periods either in buffer (controls) or in buffered vinblastine. The procedure was repeated 5 times.

Electron microscopy

Cells were fixed with a freshly prepared mixture of 1 % OsO_4 and 4 % glutaraldehyde (1:1) in 0.1 M sodium cacodylate buffer, pH 7.4, for one hour at 0-4°C (Laiho et al., 1971). They were then stained with 1 % uranyl acetate in water en block, dehydrated in a graded series of ethanol and embedded in LX-112 (Ladd). Thin sections were cut with diamond knives on Sorval Porter-Blum MT-1 and Reichert-Jung Ultracut ultramicrotomes, stained on grids with uranyl acetate and lead citrate and examined with a JEM 100-U microscope.

Morphometric analysis

Ultrathin sections, having an interference colour of silver to grey (about 600-900 Å thick), were cut from each block. 10 micrographs, representing one experiment, were taken at random at 5300 x primary magnification from the best

section of each block. Since the procedure was repeated 5 times, a total of 50 electron micrographs were taken from each test point. The morphometric analysis was performed by point counting (Weibel, 1969) and the test lattice used was the double-square lattice described by Weibel and Bolender (1973). Stereological estimates were expressed as densities of volume or numbers of AV's and related to cellular volume.

Standard methods were used to calculate means, standard errors and standard deviations. Student's t-test was used for testing significance between the means.

RESULTS

Control cells which were incubated in buffer alone had a large concave nucleus, many free ribosomes in the cytoplasm and very few lysosomes and AV. The Golgi apparatus was prominent but the endoplasmic reticulum was scanty. The volume density of AV's increased significantly ($p<0.050$) after only 3 min incubation with vinblastine. The significant difference between the vinblastine-incubated and control cells persisted after 30 min ($p<0.010$), 1 h ($p<0.001$) and 2 h ($p<0.010$) (Fig. 1).

The significant increase in the volume density of AV's was mainly due to the significant increase in their number (Table 1). The AV's in both the control and vinblastine-incubated cells contained all the cellular components except nuclear material. The engulfed material was recognizable in the apparently newly formed AV's which were limited by double, smooth membranes. The older AV's were limited by a single smooth membrane and the engulfed material was at different stages of degradation. The appearance of AV's as well as the other changes induced by vinblastine were similar to those described earlier (Hirsimäki et al., 1975).

TABLE 1. Numerical densities (N_V) of autophagic vacuoles in Ehrlich ascites cells 3 min, 30 min, 1 h and 2 h after vinblastine (VBL; 10^{-4} M) incubation as compared with controls.

			Incubation time			
Symbol	Dimension	Treatment	3 min	30 min	1 h	2 h
N_{VAV}	$10^6 \cdot / \mu m^3$	Control	0.003±0.001	0.002±0.001	0.001±0.0002	0.004±0.001
		VBL	0.012±0.004*	0.016±0.004**	0.018±0.002***	0.025±0.005**

Values given are means from five experiments ± standard error. Levels of significance compared with controls: *p<0.050, **p<0.010, ***p<0.001

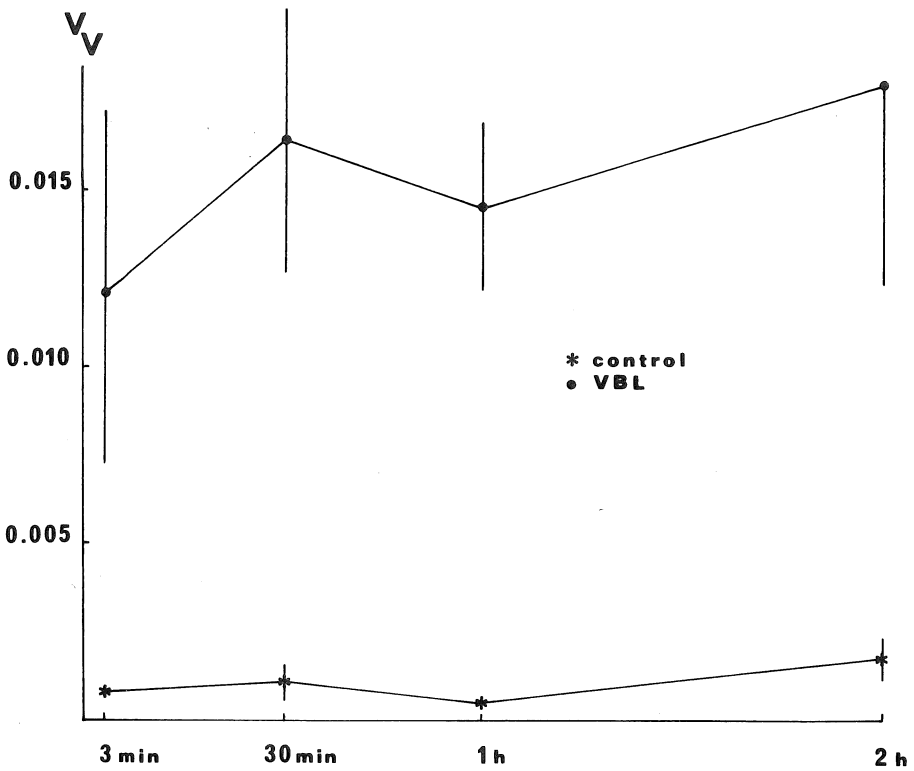

Fig. 1. Volume densities (V_V; um^3/um^3) of autophagic vacuoles at various stages of development in Ehrlich ascites cells after incubation with 10^{-4} M vinblastine. Each symbol is the mean value from five experiments and the bars indicate the standard error.

DISCUSSION

The results of the present study clearly showed that incubation of Ehrlich ascites cells in medium containing vinblastine induced a significant increase in the number of AV's for at least 2 h. Accumulation of AV's has been described in a variety of cells treated with vinblastine both in vivo and in vitro (Arstila et al., 1974; Hirsimäki et al., 1975; Hirsimäki et al., 1976; Nevalainen, 1975). On the basis of the result that vinblastine inhibits intracellular protein breakdown (Kovács et al., 1982), it has been suggested that vinblastine impairs the fusion between lysosomes and AV's,

resulting in a retarded turnover of AV's and hence to their accumulation in the cytoplasm (Kovács, 1983). However, the experimental periods in the earlier experiments were rather long. For example Kovács (1983) used a 2 h experimental period in a quantitative study of vinblastine-induced autophagocytosis in mouse seminal vesicle cells in vivo. In the present study the first specimens for electron microscopy were taken after only 3 min incubation with vinblastine, but a significant increase in the numerical density of AV's was already evident. Since the average half-life of an AV has been estimated as 8-9 min, at least in the liver (Mortimore and Schworer, 1977; Pfeifer, 1978), the rapid significant increase in the numerical density of AV's suggests that vinblastine also accelerates the rate of AV formation. Thus the experimental model presented offers a useful tool for studies on the formation of AV's.

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REFERENCES

Arstila AU, Nuuja IJM, Trump BF. Studies on cellular autophagocytosis. Vinblastine-induced autophagy in the rat liver. Exp Cell Res 1974: 87: 249-252.

Arstila AU, Trump BF. Studies on cellular autophagocytosis. The formation of autophagic vacuoles in the liver after glucagon administration. Amer J Pathol 1968: 53: 687-733.

Hirsimäki P, Pilström L. Studies on vinblastine-induced autophagocytosis in mouse liver. III. A quantitative study. Virchows Arch (Cell Pathol) 1982: 41: 51-66.

Hirsimäki P, Trump BF, Arstila AU. Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. Virchows Arch (Cell Pathol) 1976: 22: 89-109.

Hirsimäki Y, Arstila AU, Trump BF. Autophagocytosis. In vitro induction by microtubule poisons. Exp Cell Res 1975: 92: 11-14.

King DW, Paulson SR, Hannaford NC, Puckett NL. The effect of injury on Ehrlich ascites tumor cell. US Army Medical Research Laboratory 1954: 170.

Kovács J. Morphometric study of the effect of leupeptin, vinblastine, estron acetate and cycloheximide on the autophagic vacuole-lysosomal compartments in mouse seminal vesicle cells. Virchows Arch (Cell Pathol) 1983: 42: 83-93.

Kovács AL, Reith A, Seglen PO. Accumulation of autophagosomes after inhibition of hepatocytic protein degradation by vinblastine, leupeptin or a lysosomotropic amine. Exp Cell Res 1982: 137: 191-201.

Laiho KU, Shelburne JD, Trump BF. Observation on cell volume mitochondrial conformation and vital-dye uptake in Ehrlich ascites tumor cells: effects of inhibiting energy production and function of the plasma membrane. Amer J Pathol 1971: 65: 203-230.

Mortimore GE, Schworer CM. Induction of autophagy by amino-acid deprivation in perfused rat liver. Nature (London) 1977: 270: 174-176.

Nevalainen TJ. Cytotoxicity of vinblastine and vincristine to pancreatic acinar cells. Virchows Arch (Cell Path) 1975: 18: 119-127.

Pfeifer U. Inhibition by insulin of the formation of autophagic vacuoles in rat liver. A morphometric approach to the kinetics of intracellular degradation by autophagy. J Cell Biol 1978: 78: 152-167.

Weibel ER. Stereological principles for morphometry in electron microscopic cytology. Int Rev Cytol 1969: 26: 235-302.

Weibel ER, Bolender RP. Stereological techniques for electron microscopic morphometry. In: Hayat MA, ed. Principles and techniques of electron microscopy, vol. 3. New York: Van Nostrand Reinhold Co, 1973: 239-296.