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Morphological evaluation of steatosis in monolayer cultures (MDCK cells) after treatment with gentamicin and valproic acid

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Summary. An attempt was made to develop a method for the morphological and morphometric evaluation of fat inclusions in monolayer cell cultures. For this purpose we used electron microscopy (SEM and TEM) and Sudan black staining methods followed by morphometric evaluation techniques. MDCK (Madin-Darby-canine-kidney) cells were treated with valproic acid (VPA; 10, 100, 1000 µg/ml medium for 22 hrs) and gentamicin (1, 10, 50 µg/ml for 60 min). Sudan black staining revealed a dose-dependent increase in fat deposits after application of the two substances. SEM inspection showed differences in the size and distribution of the fat inclusions. TEM findings only partly allowed us to differentiate clearly between VPA- and gentamicin-induced fat inclusions.

Key words: Steatosis in vitro, MDCK cells, Gentamicin, Valproic acid

Introduction

In vitro systems are used to an ever increasing extent for the demonstration of toxic effects (Goldberg, 1986). These systems have their advantages as well as disadvantages. On the one hand, most of the *in vitro* techniques only allow the investigation of certain less complex problems and the obtained findings are in most cases clear-cut. This, however, involves the problem of an applicability of the results obtained *in vitro* to the human situation (Ekwall, 1980; Johnson and Kochhar, 1983; Bardlaw, 1986; Stark et al., 1986). One *in vitro* model simulates only certain aspects of the whole spectrum of *in vivo* functions and is, therefore, only suitable for the investigation of certain problems (Merker et al., 1980, 1981). Hence, a variety of *in vitro* systems is required to test the whole spectrum of in vivo functions *in vitro*. Thus, the capabilities and functions of an *in vitro* system must be known to appropriately employ it for substance testing. A critical analysis of the full range of available *in vitro* systems shows that there are still some gaps or the available methods are not yet sophisticated enough (Rowan, 1984). This also applies to the quantitative evaluation of fat deposits with morphological means. The biochemical measurement of lipid and lipoid deposits is rather time-consuming and requires great amounts of cells (Kates, 1986). It is, therefore, not so suitable for the screening of substances. Thus, using morphological techniques we tried to quantitatively and qualitatively evaluate the deposition of lipids and lipoids in simple monolayer cultures after application of valproic acid and gentamicin. *In vivo*, valproic acid causes steatosis of liver and kidney cells via a still largely unknown mechanism (Gerard et al., 1984; Nau et al., 1984; Graf et al., 1985; Eadie et al., 1988). Gentamicin, however, leads to an increased deposition of lipoids in kidney cells via an inhibition of lysosomal enzymes, especially lipases (Laurent et al., 1982).

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Materials and methods

Cell Culture. MDCK cells (Madin-Darbyn-canine-kidney: Gibco/Bethesda Research Laboratories) grown in Ham's F12 medium (addition: 10% FCS, 1% of each streptomycin, penicillin, glutamine) served for the experiments. The cells were grown at a density of 5×10^5 on glass and Thermanox cover slips in plastic dishes of 6 cm in diameter. The substances were added on the fourth day. At this stage large cell colonies have already formed. Gentamicin was added at concentrations of 1, 10 and 50 µg/ml medium and valproic acid at 10, 100 and 1000 µg/ml medium. After an exposure period of 60 min (gentamicin) and 22 hrs (valproic acid) the cells were prepared for TEM and SEM or stained with Sudan black. Exposure periods and concentrations had been established in preliminary experiments according to Nau et al. (1984) and Laurent et al. (1982).

Sudan Black Staining. This staining method serves above all to stain neutral lipids, but also other lipids. Ethanol (70%) was added to an 0.1% Sudan black-B solution (Serva, Heidelberg, FRG). These glass cover slips together with the cells were rinsed three times with PBS buffer. Subsequently, the cover slips were placed into dishes and covered with Sudan black solution. After 30 min the staining material was removed by repeated rinsing with PBS until the solution in the dishes was clear. Subsequently, the glass cover slips were mounted onto specimen holders and embedded in glycerin.

Morphometric Evaluation. Relative quantification of the staining intensity of the lipids was performed on a microscope (Zeiss, × 250 with oil) using an image analysis system (IBAS II, Kontron). Certain grey values were determined which served to demonstrate particles stained with Sudan black. At first the amount of Sudan black-positive inclusions was determined per cell and measurement (= «counts»). The area of these inclusions was the portion of grey areas in the measured areas of a cell (= area%). The average area of one fat inclusion (μm^2) is the grey area of all inclusions of a cell divided by their counts.

The measured values represent the mean values ± standard deviations (+/–) of 10 individual measurements each. The significance of the differences among the measured values was determined according to the Student's t-test.

Transmission Electron Microscopy. The monolayer cells on the Thermanox cover slips were fixed for 1 hr in Karnovsky's solution (3% paraformaldehyde plus 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2) and postfixed for another hour in cacodylate-buffered 1% OsO_4 at ph 7.2. Subsequently, they were washed in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone (30, 50, 70, 100%) and embedded in Mikropal (Ferak, Berlin, FRG). Thin sections were prepared using a Reichert OmU2. Contrasting with uranyl acetate and lead citrate (Reynolds, 1963) was followed by electron microscopic inspection (Zeiss EM 10 and 109).

Scanning Electron Microscopy. The cells grown on glass cover slips were fixed in Karnovsky's solution and OsO_4 (see TEM). Dehydration was performed in the ascending alcohol series up to absolute alcohol. Critical point drying was carried out with CO_2. The preparations were sputtered with gold palladium (thickness of the layer approximately 300 Å). This was followed by inspection on a Zeiss SEM (ISI 100).

Results

After Sudan black staining small lipid inclusions were irregularly distributed over control cells. Thus, morphometric evaluation using an IBAS instrument only revealed a small grey area of 18.7%, but many counts per cell (48) according to the great number of small lipid inclusions. From this small average, area per lipid inclusion (1.1 μm^2) could be calculated. Treatment with gentamicin and valproic acid changed these data. Application of 10 and 50 µg/ml gentamicin increased the number of lipid inclusions that were distributed over the whole cell. After these concentrations the grey area increased to 25.6% and 38.4% as compared with the controls. Despite many counts per cell (29 and 41) the average area of a lipid inclusion increased (2.1 and 3.2 μm^2). However, the results obtained after 1 µg/ml gentamicin were almost identical with the control (Table 1, Fig. 1).

The results after different concentrations of valproic acid showed a decrease in the number of Sudan black-positive inclusions (20/20/12). In contrast, the grey area per cell (23.6 - 51.8%) increased. This yielded, an average area of lipid inclusions of 2.8 - 11.2 μm^2. Hence, the increase in the latter parameter was due to an increase in the size of lipid inclusions (Table 1, Fig. 1).

Graphic representation of the percentage of grey area per cell shows a parallel, dose-dependent curve after application of the two substances (Fig. 2). A linear increase after gentamicin was observed for the average area of lipid inclusions (Fig. 3). After the highest concentrations of valproic acid, however, the curve was much steeper than after gentamicin.

Thus, it follows that gentamicin as well as valproic acid caused a strong dose-dependent increase of lipid inclusions in MDCK-cells, whereby valproic acid induced stronger effects and larger lipid inclusions.

Transmission Electron Microscopy. TEM inspection revealed a variety of lipid inclusions of up to 0.5 µm in size. All transitional forms from granules with content to empty vacuoles were observed. Thus, part of the inclusions corresponded to typical deposits of neutral lipids in other cell types. These inclusions were characterized by a homogeneous content of distinct electron density (Fig. 5B). A bordering membrane could clearly be demonstrated. Other inclusions were surrounded by a bright structure less zone (Fig. 4E). Often the dense content was excentrically located in such vacuoles. At one side they touched the bordering membrane, whereas at the other side a crescent-shaped halo remained (Fig. 4B). Moreover, vacuoles freed from lipids during dehydration were seen that did not show any content (Figs. 4B, C, D). Despite the evaluation of a great number of cells TEM inspection did not reveal a clear-cut difference between granules after gentamicin and valproic acid treatment. Other types of inclusions were lamella-like structures which contained irregularly arranged concentrically layered membranes (Fig. 5A). But these myelin-like inclusions were rare in both experimental series.

Scanning Electron Microscopy. SEM inspection showed lipid inclusions in the form of spherical elevations. After treatment with the two substances such elevations could clearly be observed. SEM inspection revealed elevations after the addition of valproic acid especially in the perinuclear region (Figs. 6B, C), whereas after gentamicin

Concentration (μg/ml)		Area %	Counts per cell	Average area of one fat inclusion (μm^2)
Control		18.7 ± 7.3	48 ± 4.1	1.1 ± 1.7
Gentamicin	1	18.6 ± 4.5	32 ± 3.0	1.1 ± 1.3
	10	25.6 ± 9.7	29 ± 2.5	2.1 ± 2.8
	50	38.4 ± 7.7*	41 ± 2.9	3.2 ± 3.6*
VPA	10	23.6 ± 4.9	20 ± 1.0	2.8 ± 2.9
	100	31.3 ± 5.7*	20 ± 0.8	3.3 ± 3.7*
	1000	51.5 ± 8.2*	12 ± 0.2	11.2 ± 11.9*

Table 1. Morphometric evaluation of Sudan black staining. The table shows the treatment of cells and the morphometric evaluation of the grey area on one cell, of the counts measured in one cell and of the average area of one fat inclusion. The asterisk (*) indicates significant results ($p < 0.05$). The values are given ± SD. Duration of treatment: gentamicin - 1 hr; VPA - 22 hrs.

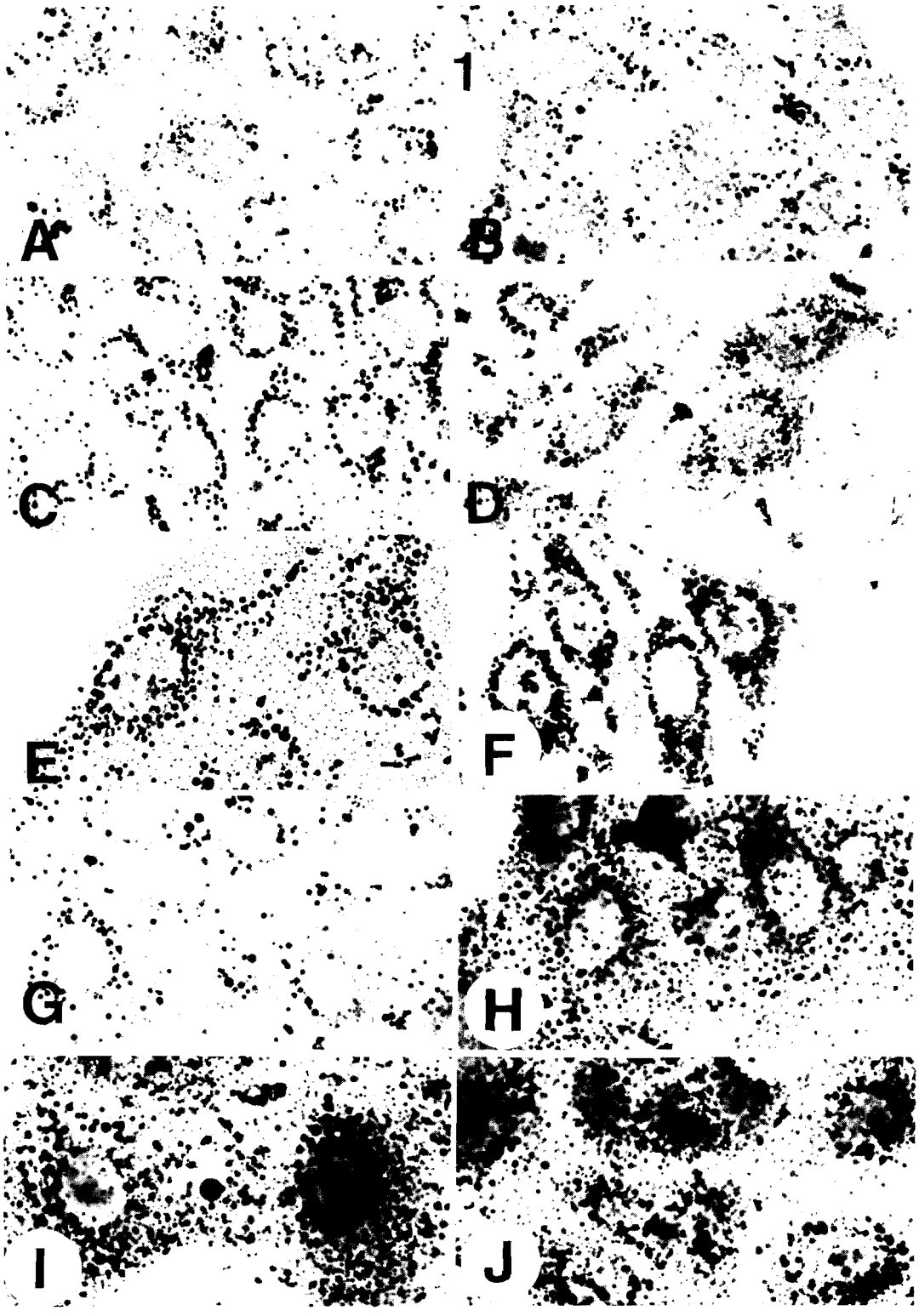

Fig. 1. Sudan black staining of MDCK cells. × 250. **A** and **B.** untreated cells. **C.** 10 μg/ml VPA for 22 hrs. **D.** 100 μg/ml VPA for 22 hrs. **E.** and **F.** 1000 μ/ml VPA for 22 hrs. **G.** 1 μg/ml gentamicin for 60 min. **H.** 10 μ/ml gentamicin for 60 min. **I** and **J.** 50 μg/ml gentamicin for 60 min.

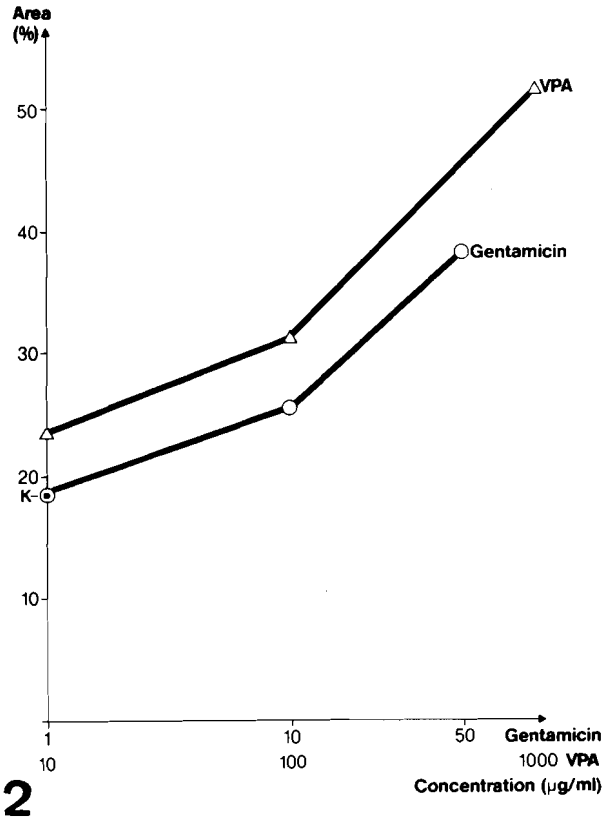

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Fig. 2. Grey area of a cell in percentage relation to the measured area (area %). Correlation between increasing concentration of the substances and increase in the grey area of a cell. K = value obtained without treatment. VPA = valproic acid.

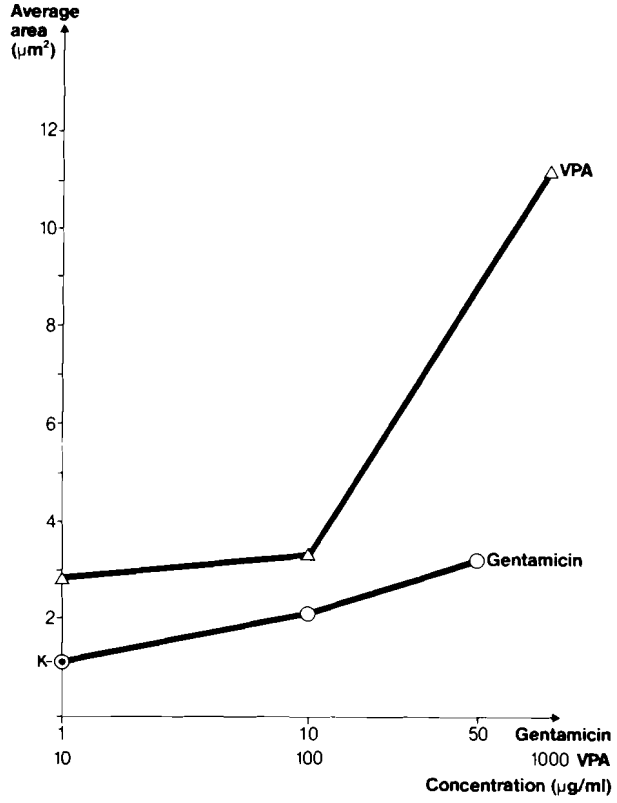

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Fig. 3. Average area (μm^2) = average area of a fat inclusion. K = value obtained without treatment. VPA = valproic acid.

they tended to be distributed over the cell. Differences in the size of the lipid inclusions were well perceptible using SEM means. After gentamicin the elevations were smaller than after valproic acid treatment (Fig. 6D).

Discussion

The Sudan black staining technique is a very reliable method for the demonstration of lipids. It does not, however, allow us to differentiate between the various lipids (neutral lipids, other lipids). Morphometric evaluation of the area/lipid inclusion yields great standard deviations. One reason for this phenomenon may be that often several subjacent and densely-packed inclusions are counted as one inclusion. This staining method and its light microscopic evaluation are easier to perform, cheaper and less time-consuming than techniques based on TEM and SEM methods.

The empty vacuoles or optically empty regions in the vacuoles seen in the TEM were certainly due to solution of the lipid content during dehydration. The occurrence of typical neutral lipid granules with homogeneous content despite dehydration might, besides differences in the preparation technique that are not very likely, speak for a differing chemical composition of the various types of inclusion.

The increase in lipid inclusions may be for two reasons. On the one hand, we might be dealing with a beginning degeneration. This is supported by our own findings. Exposure to valproic acid for more than 25 hours strongly changed the cells and they assumed a rounded shape. Similar changes were observed when the cells were exposed to gentamicin for more than 3 hours.

Fig. 4. TEM: **A.** MDCK cells, untreated. × 6,000. **B.** MDCK cell, 100 µg/ml VPA for 22 hrs. × 15,000. **C.** MDCK cell, 100 µg/ml VPA for 22 hrs. × 10,000. **D.** MDCK cell, 10 µg/ml gentamicin for 60 min. × 15,000. **E.** MDCK cell, 50 µg/ml gentamicin for 60 min. × 27,000. Various types of fat inclusions in the treated cells showing content (*) and without content (X).

Fig. 5. TEM: **A.** MDCK cell, 50 µg/ml gentamicin for 60 min. A lamellar body-like inclusion (arrow); fat vacuoles (*); I = intercellular space. × 27,000. **B.** MDCK cell, 1000 µg/ml VPA for 22 hrs. Numerous large and typical fat inclusions (*). × 50,000

Fig. 6. SEM: **A.** MDCK cell, untreated, flat cells with only few and very small fat inclusions (arrow). × 400. **B.** MDCK cells, 100 µg/ml VPA for 22 hrs. Clear-cut increase in fat inclusions in the perinuclear region (arrow). × 400. **C.** MDCK cell, 100 µg/ml VPA. * = nucleoli. × 3,000. **D.** MDCK cell, 50 µg/ml gentamicin for 60 min. Numerous diffuse fat inclusions (arrow). * = nucleoli. × 1,500

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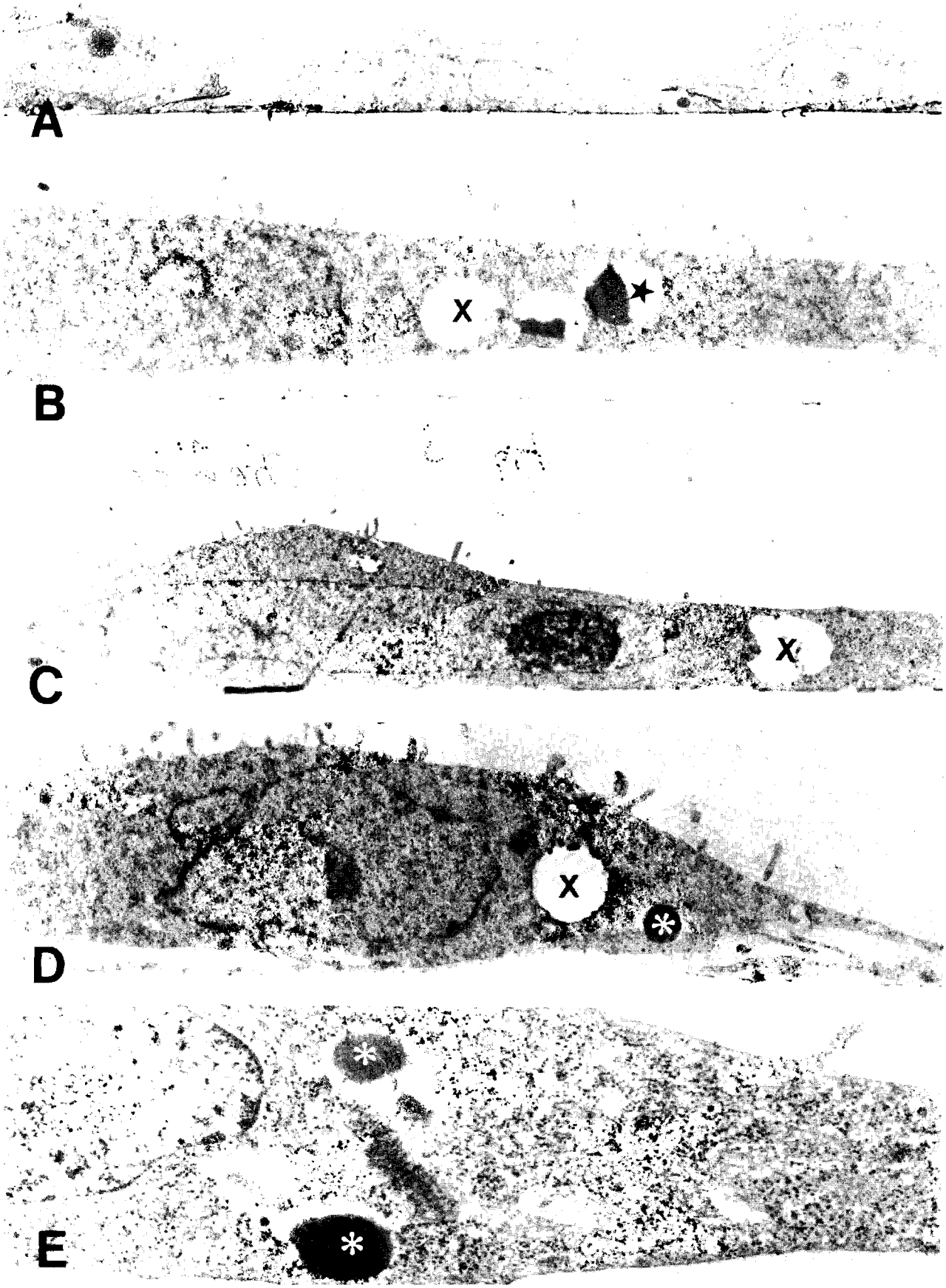

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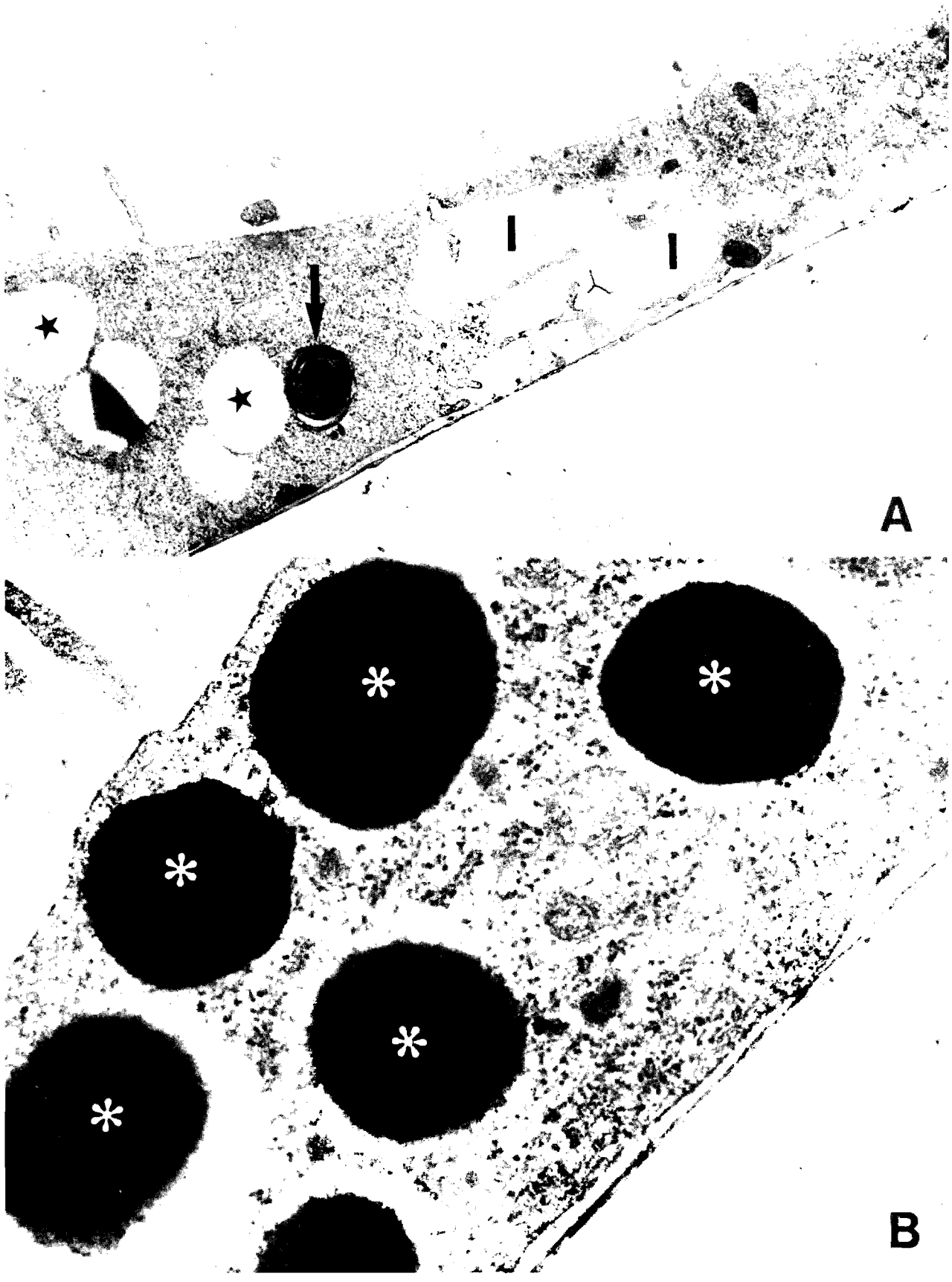

A
B
D
C

On the other hand, the lipid inclusions, shortly after exposure, might also point to the mode of action of the applied substances without having a cytotoxic effect at the applied doses and exposure time.

Gentamicin is known to inhibit the activity of the lysosomal enzymes spingomyelinase and phospholipase A, since it induces an accumulation of phospholipids in the lysosomes (Aubert-Tulkens et al., 1979; Laurent et al., 1982). The occurrence of lamella-like bodies might speak for such a mechanism. Valproic acid is disaggregated by ß-oxidation and excreted in the liver after glucoronidation, thus interfering with ß-oxidation (Coudé et al., 1983; Jezequel et al., 1984; Horie and Suga, 1985).

Given a disturbed ß-oxidation, the occurrence of lipid inclusions would be quite feasible. Valproic acid and its derivatives, e.g. 4-en-VPA, also led to morphological changes, such as occurrence of lipid inclusions, myelin bodies and mitochondrial abnormalities, *in vivo* in the liver of the mouse, rat and man (Berthelot, 1982; Zimmermann and Ishak, 1982; Kesterson et al., 1984; Nau et al., 1984).

These findings are in agreement with our results obtained with MDCK-cells in monolayer culture. Here, a correlation exists between *in vivo* and *in vitro* experiments under the influence of valproic acid and gentamicin.

Hence, Sudan black staining and subsequent morphometric evaluation appear to be suitable for the demonstration of substances that damage cells and induce steatosis. This might enable us to make statements on the toxicity and modes of action *in vitro.*

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