Perhexiline maleate toxicity on human liver cell lines

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SUMMARY When added to the culture medium of human liver cell lines, perhexiline maleate induced formation of numerous myeloid bodies containing unicentric or multicentric smooth membranes within a few days. The nine lysosomal enzyme activities studied, except for β-galactosidase which decreased, remained unchanged. These results indicate that on cultured human liver cells perhexiline maleate has an effect similar to that described on hepatocytes of some patients treated with this drug and suggest that myeloid body formation is not due to impairment of lysosomal enzyme activities.

Since 1973 perhexiline maleate has been often prescribed as an antianginal drug. Various side-effects related to its use have been described, particularly hypoglycaemia,1 2 massive weight loss,3 neuro-myopathy,4 6 and hepatopathy.4 7 The frequency of hepatopathies seems to be low.13 However, some abnormal liver functions, such as an increase in the level of serum transaminase, might be seen in about 50% of cases.14

Histological study of liver tissue shows damage of which is correlated with the severity of clinical and biological disorders; major damage includes portal and septal sclerosis, moderate inflammatory infiltrate, and megalocytic and necrotic hepatocytes.15 17 Under the electron microscope, the most characteristic features are the occurrence of Mallory bodies similar to those reported in acute alcoholic hepatitis, giant mitochondria, and, in particular, numerous abnormal lysosomes similar to voluminous membrane-limited inclusions with concentric smooth membranes.10 16 Such inclusions are also found in patients suffering from neuropathy, in peripheral nerves18 19 as well as in cutaneous and muscular tissues.11 Administration of perhexiline maleate to the rat is followed by similar cellular alterations in various organs with the exception of the liver.20 The mechanism of the drug's toxicity remains unknown. Moreover, whether or not this drug has a direct effect on liver cells has often been discussed. In order to answer these questions we studied the toxicity of perhexiline maleate on human liver cell lines.

METHODS

LIVER CELL CULTURES

Five liver cell lines were used. One was obtained from a 4 month old fetus after spontaneous abortion; the second was obtained surgically from a 3 day old child and the other three surgically from 30, 40 and 44 year old adults suffering from non-hepatic disease. Liver fragments were cultured in plastic flasks using Eagle's medium, 10% human serum, and 95% air—5% CO₂ gas mixture.21 Subcultivation was achieved with 0.1 M trypsin. The liver cell lines were cultured in Eagle's medium supplemented with 10% fetal calf serum.22 Cell lines were used between the fifth and the 15th passage. Perhexiline maleate (Pexid) (Laboratoires Merrell-Toraude, Paris) was added to a final concentration in the medium between 1 and 20 μg/ml. Perhexiline maleate was added either four hours after seeding and four days later with medium renewal or after the cells had reached confluence and was renewed every 24 hours. In some experiments, after three days in the presence of the drug, liver cell cultures were replenished with normal medium for eight days, with or without further subcultivation.

CELL COUNTS

Cells were plated in plastic cultures dishes at 1 × 10⁶ cells/ml, 2 ml/dish, and counted with a couler counter (Coultronics, Andilly, France) after one, two, three, and seven days of culture. The medium was renewed on the fourth day.

ULTRASTRUCTURAL STUDIES

For the ultrastructural studies, cultures were fixed...
in situ in a 2.5% glutaraldehyde solution buffered to pH 7.2 with 0.1 M sodium cacodylate for 10 minutes at -4°C and post-fixed afterwards in a 1% osmium tetroxide solution buffered with 0.1 M sodium cacodylate for 30 minutes. Dehydration was effected by passing the sample through ethanol and the cells were embedded in Epon. Ultra-thin sections were doubly stained with uranyl acetate and lead citrate and examined with an electron microscope (Hitachi HU 11E IF).

For stereological analysis, cultures treated for four days in the presence of 2 μg/ml perhexiline maleate were compared with control cultures. A total of 25 electron micrographs were taken at random at an original magnification of ×5000 in each culture. The analysis of the micrographs was performed by projection on a screen and the relative volume of the myeloid bodies was calculated according to Weibel. The statistical analysis was made using Student's t test.

HISTOCHEMICAL LOCALISATION OF ACID PHOSPHATASE
For demonstration of acid phosphatase activity, β-glycerophosphate was employed as substrate. Control tests were carried out with sodium fluoride (10 nM).

LYSOSOMAL ENZYME ASSAYS
Lysosomal enzyme activities were measured in cell pellets and after direct incubation of perhexiline maleate with lysosomal enzyme solutions from crude or purified liver cell extracts. Purification was achieved by chromatography on a concanavalin A-sepharose column in 10 mM sodium chloride, 10 mM phosphate buffer, pH 7, followed by elution

**Fig. 1** Number of cells as a function of culture time in control (□□□□) and in perhexiline maleate-treated (2 μg/ml, ●●●●; 5 μg/ml, ■■■■) cultures. The cells were seeded at 1 x 10⁶ cells/ml, 2 ml/dish. The drug was added after four hours and four days with medium renewal. Mean of three experiments.

**Fig. 2** Control liver cell at confluency (10th passage); the cytoplasm contains dilated rough endoplasmic reticulum (RER), elongated mitochondria (M), and some lysosomes (L). Nucleus, N; Golgi apparatus, G. X 11 000. (Original magnification in this and the following legends.)
with a 0.75 M α-D mannose solution in 500 mM sodium chloride, 10 mM phosphate buffer, pH 7 at 37°C.

Cells were washed in situ with phosphate buffer saline then collected by scraping with a rubber policeman. Cells pellets were resuspended in one ml of 0.01% Triton X 100, then lysed by sonication (20 KHz, 20 W) twice for 30 seconds at 0°C. Protein estimations were carried out according to the method of Lowry et al. and DNA was determined according to Munro and Fleck.

Arylsulphatase A activity was assayed according to the method of Baum et al. For the eight other lysosomal enzyme activities (α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, α-D-glucosidase, β-D-glucuronidase, hexosaminidase, α-D-mannosidase, acid phosphatase), concentration in substrate, buffers, and conditions of incubation were those described by Troost et al. for α-L-fucosidase, 1 mM p-nitrophenyl-α-L-fucopyranoside, 0.1 M sodium acetate buffer, pH 5.5; for α-D-galactosidase, 5 mM p-nitrophenyl-α-D-galactopyranoside, 0.1 M citrate buffer, pH 4.8; for β-D-galactosidase, 0.5 mM p-nitrophenyl-β-D-galactopyranoside 0.1 M citrate buffer, pH 3.6; for α-D-galactosidase, 2 mM p-nitrophenyl-α-D-glucopyranoside, 0.1 M citrate buffer, pH 3.5; for hexosaminidase, 1.25 mM p-nitrophenyl-2-acetamide-2 deoxy-β-D-glucopyranoside, 0.1 M citrate buffer pH 4.3, 0.3 M sodium chloride; for α-D-mannosidase, 1.5 mM p-nitrophenyl-α-D-mannopyranoside, 0.1 M citrate buffer, pH 4.6, 3 mM zinc chloride; for acid phosphatase, 1 mM p-nitrophenyl phosphate, 0.1 M sodium acetate, pH 5. The values of lysosomal enzyme activities were expressed in nkat per g protein. Statistical analysis was performed according to Mann and Whitney's test.

**Results**

Effects of perhexiline maleate on cell growth, cell ultrastructure and lysosomal enzyme activities were similar in human cell lines derived from fetal, newborn, and adult liver tissue.

**CELL GROWTH**

After 24 to 48 hours in the presence of perhexiline maleate at concentrations above 10 μg/ml, most of the cells became detached from the substratum and died. At lower drug concentrations ranging between 5 and 10 μg/ml, cell growth was partly inhibited, and under phase-contrast microscopy dense granules were visible within 24 hours in perinuclear areas. The number of these inclusions increased during the following days and some lipid droplets were also observed. In the presence of 2 μg/ml perhexiline maleate, cell growth was not altered (Fig. 1); the mean time between two consecutive passages was similar to that found with control cultures. When cells were replenished with normal medium after three days of treatment with perhexiline maleate, they still contained numerous cytoplasmic inclusions...
eight days later even after they had been trypsinized and subcultivated.

**CELL ULTRASTRUCTURE**

As shown in Figs 2 and 3, no evident changes appeared in control cells during four days at confluency. The cells contained elongated mitochondria, well-developed Golgi apparatus, partly dilated rough endoplasmic reticulum, and some secondary lysosomes. A few of them had concentric smooth membranes. In the presence of perhexiline maleate, the major ultrastructural alteration of human liver cells was the rapid appearance of cytoplasmic inclusions (Fig. 4). With drug concentrations ranging between 1 and 5 μg/ml, some inclusions were observed within 24 hours. Their number and size increased during the following days and reached a maximum between four and six days. The other organelles were not altered.

The cytoplasmic inclusions were typical myeloid bodies, appearing as membrane-limited structures and containing unicentric or pluricentric smooth membranes (Figs 4 and 5). These myeloid bodies were usually round but they had sometimes a rectangular shape (Fig. 5). A few inclusions contained lipid material (Fig. 5) or smooth membranes in reticular arrangement (Fig. 6). The latter were seen only after the fourth day of drug treatment. Mye-

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**Fig. 4**  Liver cell treated with perhexiline maleate at the concentration of 2 μg/ml for two days (10th passage): the cytoplasm contains numerous myeloid bodies with unicentric (arrow) or multicentric (double arrow) smooth membranes. Lipid material (*) is observed in some inclusions. Rough endoplasmic reticulum (RER) and mitochondria (M) are not altered. Nucleus, N. × 9000.

**Fig. 5**  Liver cell treated with perhexiline maleate at the concentration of 2 μg/ml for three days (10th passage). Note the polymorphism of myeloid bodies. Some inclusions show a rectangular shape (arrow). × 13,600.
Loid bodies remained numerous and were morphologically unmodified in cells cultured for eight days in normal medium after three days of treatment with perhexiline maleate (Fig. 7).

Stereological analysis showed that the relative volume of inclusions containing concentric smooth membranes per unit of liver cell cytoplasm increased 20-fold in cells cultured for four days in the presence of 2 µg/ml perhexiline maleate. The difference between control (1·1 ± 0·3) and treated-cells (21·7 ± 2·1) was significant (P < 0·001). The relative volume of the inclusions was not significantly changed in control cells after four days at confluency.

ACID PHOSPHATASE LOCALISATION
As shown in Figs 8 and 9, the staining for acid phosphatase activity was quite similar in control and in PM-treated cells.

LYSOSOMAL ENZYME ACTIVITIES
Since the ratio mg proteins/mg DNA was not significantly different in control cells and in cells cultured in the presence of 2 µg/ml PM for four days (8·22 ± 0·3 versus 8·44 ± 0·8), lysosomal enzyme activities were expressed/g protein. The data are summarised in the Table. Only β-D-galactosidase activity was significantly modified and showed a

Fig. 6 Liver cell treated with perhexiline maleate at the concentration of 2 µg/ml for six days (10th passage); some myeloid bodies contain smooth membranes in a reticular arrangement (arrow). × 18 400.

Fig. 7 Liver cell treated with perhexiline maleate at the concentration of 2 µg/ml for three days then cultured in normal medium for eight days (10th passage). As in Fig. 2 numerous myeloid bodies with unicentric or multicentric smooth membranes are observed. × 18 400.
Fig. 8  Localisation of acid phosphatase in control cells. ×120.

Fig. 9  Localisation of acid phosphatase in cells treated with perhexiline maleate at the concentration of 2 μg/ml for four days. ×120.

decrease of 30%. No significant change was found in the eight other hydrolase activities. In the presence of PM at the concentration of 5 μg/ml, β-galactosidase activity was also decreased (p < 0.001) but the three other enzymes tested (hexosaminidase, α-mannosidase, acid phosphatase) remained unchanged.

On the other hand, after incubation of PM at concentrations ranging from 2 to 4 × 10^9 μg/ml with lysosomal enzyme solutions from crude or purified liver cell extracts, for one hour at 37°C, no modification of lysosomal enzyme activities, including that of β-D-galactosidase, was found.

Discussion

Ultrastructural examination of human liver cells, cultured in the presence of perhexiline maleate, revealed peculiar alterations, consisting in numerous cytoplasmic bodies and some lipid droplets. Other organelles remained unchanged. These cytoplasmic inclusions were mainly of the myeloid body type. Cytochemical studies showed that these inclusions belong to the group of lysosomeŝ and, in cultures from mouse spinal ganglia. Perhexiline maleate toxicity was rapid and direct on cultured human liver cells. Contrary to some authors' conclusions, this toxicity does not require to be potentiated by other xenobiotics, such as alcohol.

Myeloid bodies contained smooth membranes in concentric and sometimes in reticular arrangement;

Table  Lysosomal enzyme activities in control and perhexiline maleate treated human liver cell lines

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>n</th>
<th>Enzyme activity (nkat per g protein)</th>
<th>Control cells</th>
<th>Treated cells</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arylsulphatase A</td>
<td>7</td>
<td>70 ± 14</td>
<td>60 ± 14</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Hexosaminidase</td>
<td>11</td>
<td>1468 ± 407</td>
<td>1384 ± 335</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>α-L-Fucosidase</td>
<td>7</td>
<td>39 ± 16</td>
<td>37 ± 13</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>α-D-Mannosidase</td>
<td>12</td>
<td>72 ± 16</td>
<td>70 ± 13</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>β-D-Galactosidase</td>
<td>9</td>
<td>69 ± 44</td>
<td>63 ± 43</td>
<td>p &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>α-D-Glucosidase</td>
<td>6</td>
<td>36 ± 7</td>
<td>39 ± 5</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>α-D-Glucuronidase</td>
<td>6</td>
<td>13 ± 4</td>
<td>16 ± 6</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>β-D-Glucuronidase</td>
<td>6</td>
<td>30 ± 13</td>
<td>31 ± 9</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>12</td>
<td>711 ± 431</td>
<td>710 ± 396</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

The figures in column 2 represent the number of cell passages tested. The values are given as the mean ± SE. The cells were cultured in the presence of PM at the concentration of 2 μg/ml for four days.
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they appeared to be the morphological support of perhexiline maleate induced-phospholipid and ganglioside storage as demonstrated by histochemical and biochemical studies.\textsuperscript{10-12} The formation of myeloid bodies is not specific to perhexiline maleate; it can be induced by a variety of drugs, including chloroquine and some other antimalarial drugs, suramin, chlorphentermine, triparanol, erythromycin, gentamycin, and coralgly. These drugs are effective on various cell types both in humans\textsuperscript{30-35} and in animals,\textsuperscript{30,31,34,37} as well as in vitro.\textsuperscript{38} In spite of their different pharmacological effects, all these drugs are cationic compounds of amphiphilic character. This could suggest a common and unspecific mechanism of their side-effect.

Several hypotheses can be put forward to explain perhexiline maleate could inhibit specific activity of one or more lysosomal enzymes. Several reports have dealt with such drug-induced alterations. It has thus been shown that sphingomyelinase activity is inhibited by gentamycin,\textsuperscript{39} cathepsin B and D and acid phosphatase activities by suramin,\textsuperscript{36} and cathepsin B activity by antimalarial drugs.\textsuperscript{39} Our results show that perhexiline maleate induced a decrease in $\beta$-galactosidase activity. As ganglioside GM\textsubscript{1}, is the natural substrate of $\beta$-galactosidase, it is attractive to correlate this decrease to intralysosomal storage of gangliosides. However, we observed no direct effect of perhexiline maleate on $\beta$-galactosidase specific activity in vitro. Furthermore, the decrease did not exceed 30\% and the eight other lysosomal enzyme activities tested remained unchanged. This decrease in $\beta$-galactosidase activity does not appear sufficient to account for the rapid accumulation of large amounts of non-hydrolysed material within lysosomes, and suggests that the physiopathological mechanism of PM intoxication is not similar to that of inherited lipid storage diseases.

On the other hand, it has been demonstrated that certain drugs accumulated up to 200-fold their serum concentration into lysosomes.\textsuperscript{39} Some of them induce storage diseases because they react with natural substrates and become unphysiological substrates for the degrading lysosomal enzymes.\textsuperscript{40} Interactions have been observed between amphiphilic drugs, such as chlorphentermine, and phospholipids by nuclear magnetic resonance.\textsuperscript{41} Formation of complexes between perhexiline maleate and polar lipids could well explain intralysosomal accumulation of concentric smooth membranes. In the same way, interactions between the drugs and lysosomal enzyme activators can occur.\textsuperscript{42}

Finally, binding of perhexiline maleate to phospholipids could result in alteration of membrane fluidity.\textsuperscript{43} Consequently the drug could induce either an increased turnover of membrane components associated with an impairment of lysosomal degradation processes or an alteration of membrane recycling as proposed by Dean.\textsuperscript{44}

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