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Changes in the Fine Structure and Function of a Hormone-Secreting Adrenocortical Tumour Investigated in Tissue Culture

D. Szabó, A. Gyévai, E. Gláz, E. Stark, M. Péteri, and O. Alánt

Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest
(Director: Prof. Dr. E. Stark)
2nd Medical Clinic, Semmelweis University of Medicine, Budapest
(Director: Prof. Dr. Gy. Petrányi)
1st Surgical Clinic, Semmelweis University of Medicine, Budapest
(Director: Prof. Dr. P. Rubányi)

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Summary. Tissue cultures of a surgically removed adrenocortical tumour causing Cushing's syndrome, and tissue cultures from the attached, tumour-free adrenal were studied. There were two cell types characteristic of tumour tissue. The cell type occurring most frequently had pronounced hypertrophied agranular endoplasmic reticulum. A fewer number of lipid-rich cells containing many electron-dense granules could also be found. The ratio of cells changed during cultivation. In the 17 days tumour culture, a higher percentage of lipid-rich cells could be observed. In spite of continuous ACTH treatment, the initially high hydrocortisone level decreased, gradually. It may be assumed that the lipid-rich cells are of reduced ability as regards hydrocortisone production.

Functioning adrenocortical tumour frequently gives rise to Cushing's syndrome. The atrophy of the attached, tumour-free, and of the contralateral cortex is a known concomitant symptom. Temporary clinical improvement can be achieved with steroid inhibitors, mainly with aminoglutethimide (Schteingart *et al.*, 1966; Fishman *et al.*, 1967; Smilo *et al.*, 1967).

The ultrastructure of adrenocortical hyperplasia and tumours, underlying Cushing's syndrome, has already been described by a fair number of authors (Holzmann and Lange, 1966; Luse, 1967; Reidbord and Fisher, 1968; Mackay, 1969; Symington, 1969; Hashida *et al.*, 1970; Macadam, 1970; Mitschke *et al.*, 1971; Urushibata, 1971; Neville and Mackay, 1972; Mitschke *et al.*, 1973; Tazaki *et al.*, 1974). The aim of the present investigation has been to find out whether or not the tumour retains its characteristic morphological and functional features in tissue culture.

Material and Methods

The right adrenocortical tumour and adrenal were removed by surgery from a 38 year-old woman. Prior to surgical intervention, the patient was clinically diagnosed to suffer from Cushing's syndrome. In addition to the conventional treatment, she was given daily doses of 0.5 g of aminoglutethimide for 6 weeks. This treatment was withdrawn 14 days prior to the operation. Immediately after exstirpation, small pieces were cut both from the tumour and the attached adrenal cortex. For polarization microscopy, sections were cut in cryostat, fixed in 4% buffered formalin. The pieces for cultivation were rinsed several times with chemically defined TC 199 medium, containing antibiotics, and then cut into pieces of about 1 mm^3.

Five separate plastic Falcon flasks, of 250 ml each, were taken and 100 to 150 such pieces were placed into each of them on a coagulate of plasma and embryonic extract. After 24 hours, the explants attached to the coagulate and began to grow. The outgrowing cells showed an epithelial-like character. Mitosis was common in the outgrowing cells. After the fifth day, the explants began to die off. The cuts were made from the outgrowing cells surrounding the explants. For electron microscopy, some pieces were placed into plastic Falcon Petri-dishes and treated in the same way as the Falcon flasks. Twenty-four hours later, the 8:2 mixture of TC 199 medium and pooled human sera were added to the cultures. In one of the Falcon flasks pieces from the attached adrenal cortex, in the other four, tissue pieces from the adrenocortical tumour were cultivated. The cultures were washed every 48 and 72 hours, resp. On the 5th day of cultivation, 100 mU/ml of ACTH (Organon, Oss.) were added to each culture. In the subsequent days nutrient medium, alternately with or without ACTH, was added to the cultures. Hydrocortisone production in the medium was determined by paper chromatography (Stark *et al.*, 1963). On the 17th day of cultivation the cultures in the Petri-dishes were processed for electron microscopy. These cultures were fixed *in situ* in 2.5% glutaraldehyde for 2 hours, post-fixed in 1% osmium tetroxide for further 2 hours. 0.1 M sodium cacodylate-HCl buffer was used to adjust the fixatives to pH 7.4. Dehydration in graded ethanol followed and the cultures in the Petri-dishes were embedded into Durcupan ACM.

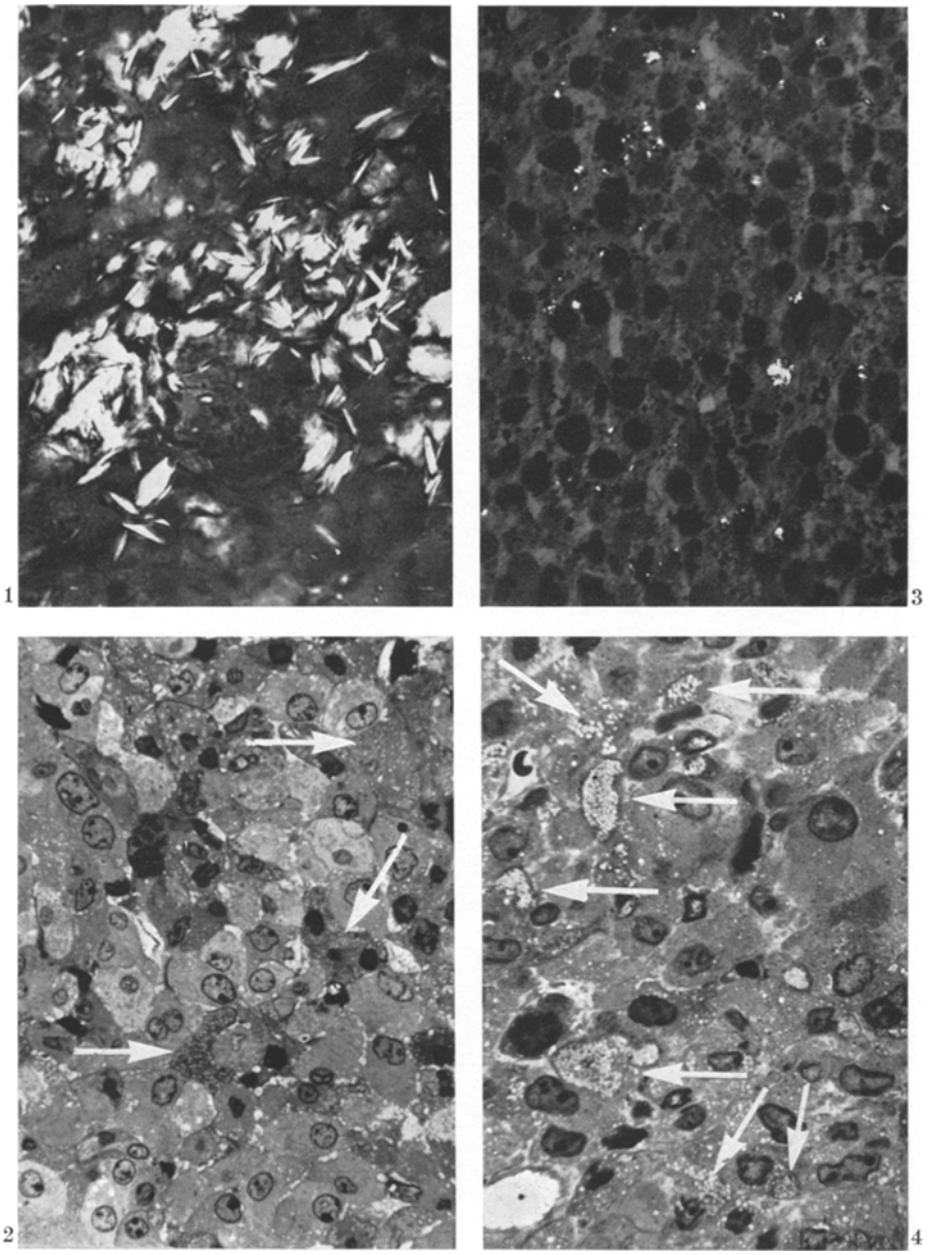
The adrenal pieces, both from the tumour and the attached uninvolved cortex, were also fixed and embedded in a similar way. Sections approximately 1 μm thick were mounted on glass slides, stained with toluidine blue and examined with light microscope. Ultrathin sections were cut on a Reichert Om U2 ultramicrotome and mounted on copper grids coated with carbon-stabilized Formvar film. The sections were counterstained first with 20% uranyl acetate, dissolved in methanol, then with lead citrate according to Reynolds (1963). The sections were studied in a JEM-6AS electron microscope, operating with 80 kV accelerating voltage.

Observations

Light Microscopic Findings. With polarization microscope, significantly more birefringent lipid was seen in the tumour-free adrenal cells than in the tumour (Figs. 1 and 2). In the zero-time tumour tissue haemorrhagic, necrotic and lobular areas occurred intermittently. The cells in the lobular areas were mainly of the fascicular type forming alveoli and cords. The tumour cells had vesicular and pleomorphic nuclei, vacuolated cytoplasm with finely dispersed lipid droplets. Sporadic mitotic activity was observed. The tumour was considered to represent an adrenocortical carcinoma (Fig. 3). In the tumour tissue cultivated for 17 days, many cells were rich in lipid. The nuclei were enlarged and vesicular in appearance (Fig. 4).

Electron Microscopic Findings. In the adrenal cortex adjacent to the tumour, the cells of the zona fasciculata were atrophied, the lipid droplets became larger and increased in number.

The morphology of the zero-time tumour tissue was highly variable. The cell type occurring most frequently had enlarged nuclei with multiple inclusions. Hypertrophy of the agranular endoplasmic reticulum (AER) was conspicuous. Some of the AER vesicles were dilated to such an extent that they became cyst-like structures. Lysosomes in the cells were less abundant. Many abnormal mitochondria can be found, they have relatively few intramitochondrial structures. The number of lipid droplets varied from cell to cell. In general, they were less numerous than normally (Fig. 5). The other characteristic cell-type observed, besides containing lipids varying in size and density, had numerous electron-dense granules mainly lysosomes. Some of these granules frequently showed a close

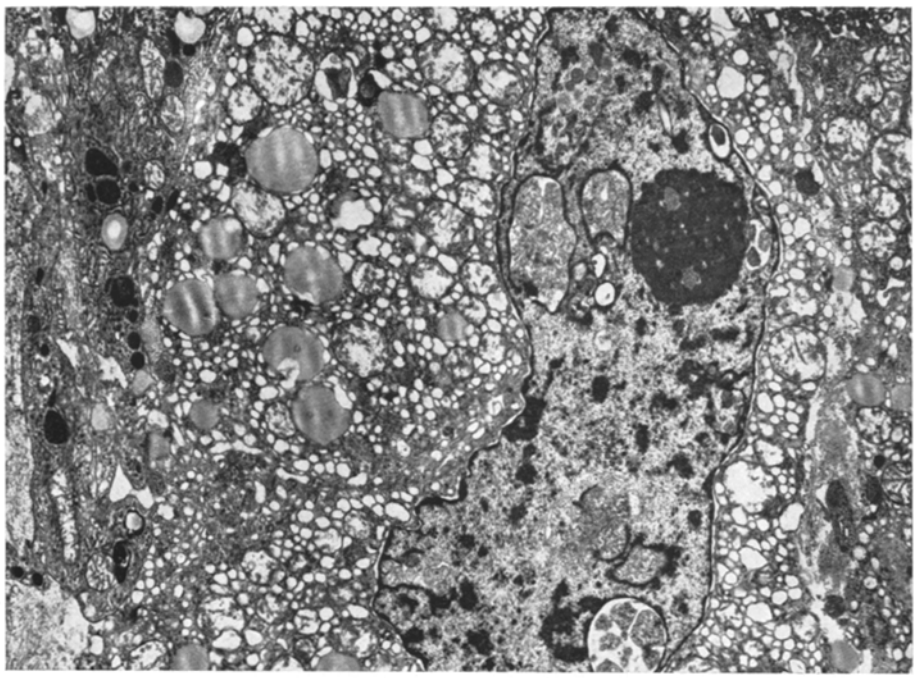


Figs. 1 and 2. Cryostat sections of adrenals in polarized light. Many birefringent lipids in the tumour-free adrenal (Fig. 1), a few in the tumour cells (Fig. 2). ×400

Figs. 3 and 4. Light micrographs of 1 μm thick sections. Stain: toluidine blue

Fig. 3. In the zero-time tumour tissue the cells show marked variation in size, shape and intensity of staining. Sporadically lipid-rich cells can be seen (arrows). ×400

Fig. 4. The tumour tissue cultivated for 17 days shows a variety of cell types. Note the great number of lipid-rich cells (arrows) and compare with Fig. 3. ×400



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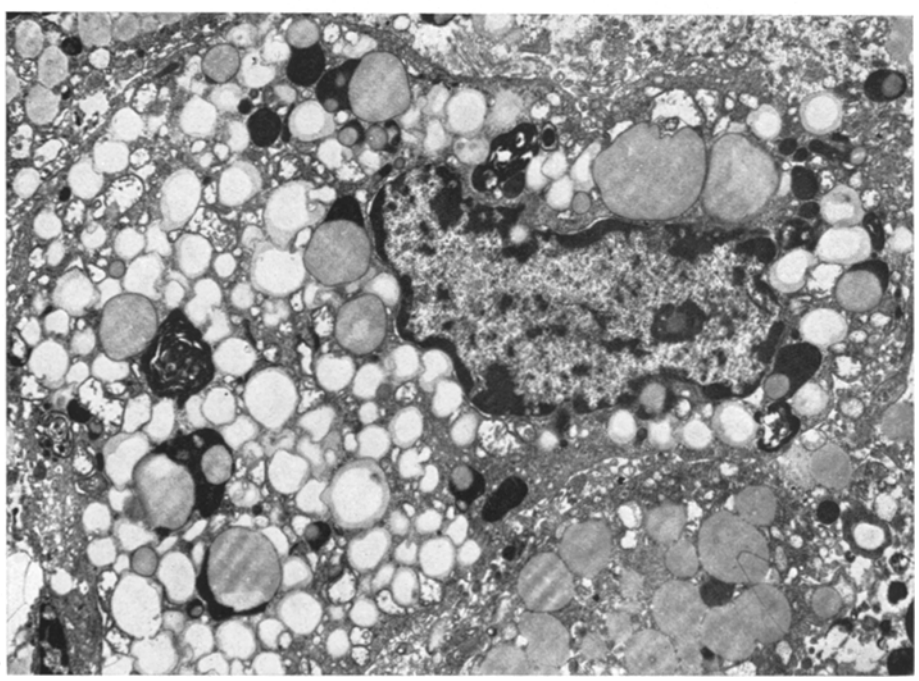

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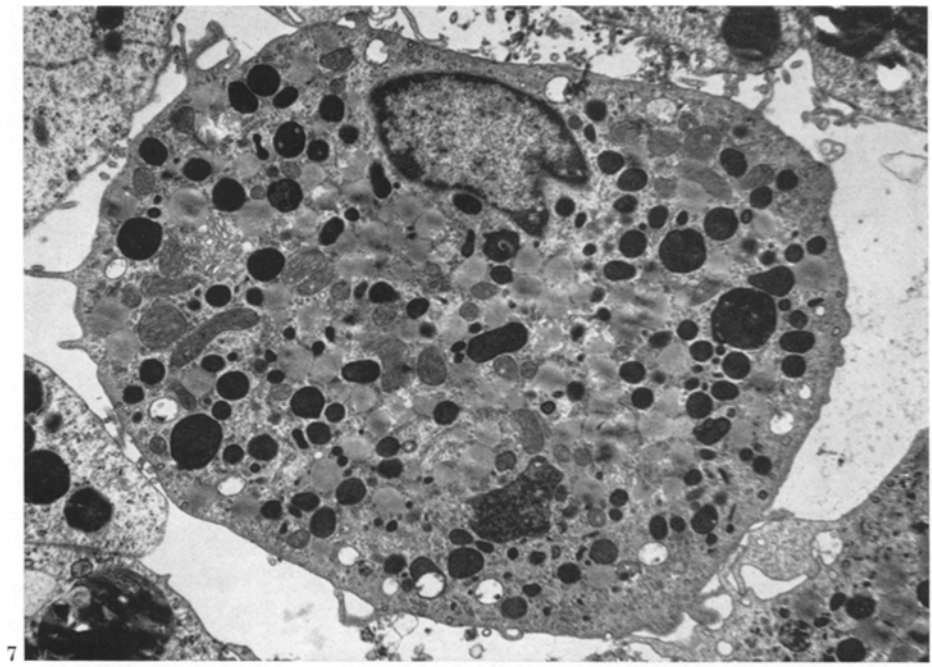
Fig. 5. Grossly enlarged nucleus with multiple inclusions, extended AER in the predominant cell type of the zero-time tumour tissue. ×7200

Fig. 6. Lipid-rich cells in the zero-time tumour tissue with numerous electron-dense bodies. ×5700



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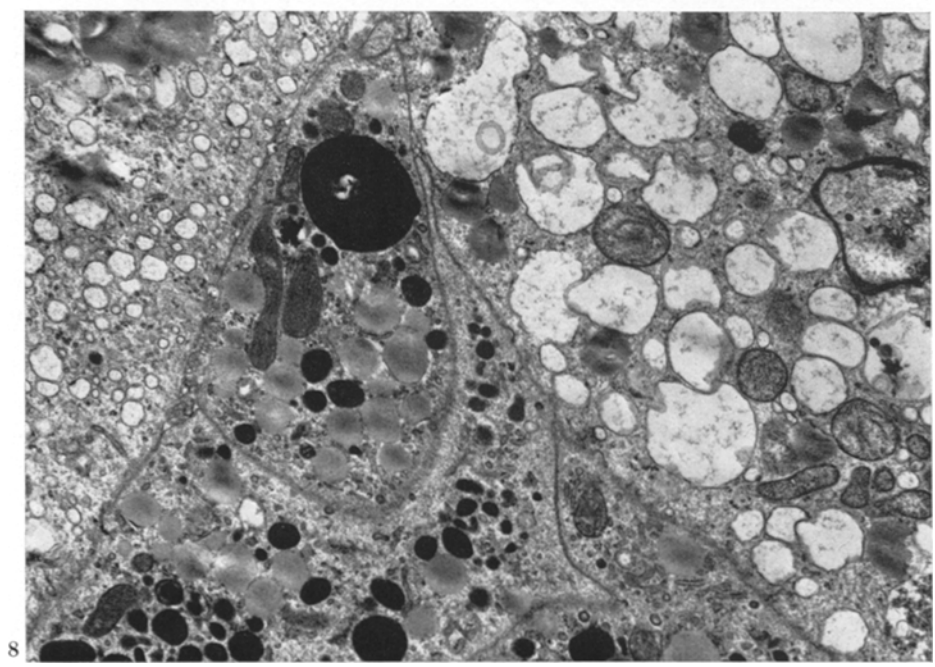

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Fig. 7. A solitary located lipid-rich cell with rounded off contours in the 17-days tumour culture. Numerous electron-dense bodies occur in the cytoplasm. ×6600

Fig. 8. Parts of cells from the 17-days tumour culture. Cells with many electron-dense bodies and lipid droplets are tightly packed between cells with abundant dilated AER. ×9800

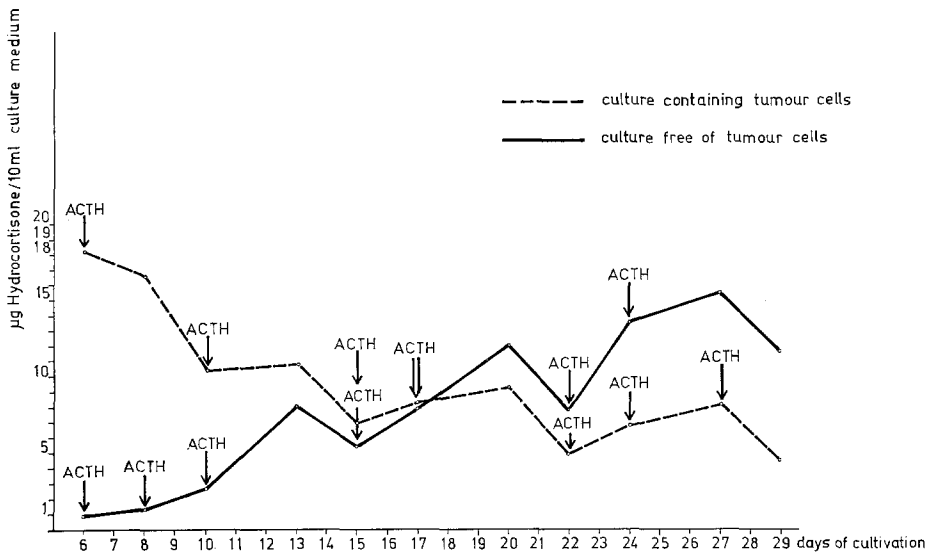

Fig. 9. ACTH-induced hydrocortisone production in adrenal tissue cultures containing tumour cells and in cultures of tumour-free adrenal fragments

structural relationship with the lipid droplets. Sporadic electron-dense bodies composed of concentrical lamellae could be seen (Fig. 6). In a lower number, cells similar to the normal adrenocortical cells also occurred in the tumour.

After cultivation for 17 days, the distribution rate of cells in the tumour was found to have changed. Cells rich mainly in lipids and electron-dense granules became predominant in the monolayer. Some of these cells with rounded off contours were located solitarily (Fig. 7), others were closely attached to the cells with hypertrophied AER (Fig. 8). Cells with hypertrophied AER were fewer in tissue culture than in the zero-time tissue. In some places the AER cisternae showed marked dilatation. A flocculent substance could be observed inside the AER cisternae. Nuclear inclusions were present also in the tissue culture. A low number of cells similar to normal adrenocortical cells were found in the tissue cultures, too.

Hydrocortisone Production. Hydrocortisone production in the tissue culture prepared from adrenocortical tumour was markedly higher than that in the culture form tumour-free tissue in the first stages of the cultivation (Fig. 9). Hydrocortisone production decreased significantly in tumour tissue cultures. It is noteworthy, that the tumour tissue culture failed to respond to ACTH.

Discussion

The presence of nuclear inclusions may give the nucleus a vesicular appearance by light microscopy. The enhanced hormone production of the tumour may lead to the atrophy of the attached adrenal cortex due to a negative feedback action. The accumulation of birefringent lipids in the atrophied cells seems to speak for

hypofunction or inactivity. Since the patient had been treated with steroid inhibitors, among others with aminoglutethimide, for rather a long time prior to surgery, the potential share of this treatment in lipid accumulation cannot be excluded. With the polarization microscope Szabó *et al.* (1974) observed similar phenomena in rats treated with aminoglutethimide and they proved this to represent accumulated cholesterol. Motlik *et al.* (1973) concluded that aminoglutethimide administration may produce alteration in human adrenals even in normal therapeutic doses.

The data of Morita *et al.* (1972) furnish evidence for the enhanced utilization of cholesterol in Cushing's syndrome. Using ^{131}I-19-cholesterol, they have demonstrated that more of cholesterol is incorporated into the adrenals of patients suffering from Cushing's syndrome than in individuals with normally functioning adrenals.

A gradual increasing hydrocortisone content in the medium, suggested the gradual regeneration of the atrophied adrenocortical cells in the zero-time tissue. These cells regained their hormone-producing ability in a few days.

The initial high hydrocortisone production in the tumour cell culture is in agreement with the abundance of the hypertrophic AER found in the zero-time tissue. Kovács *et al.* (1974) observed similar ultrastructural phenomena in the aldosterone-secreting adrenocortical adenoma. The AER enzyme system is involved in many steps of steroid biosynthesis, such as cholesterol formation from acetate, the conversion of pregnenolone to progesterone and the transformation of progesterone to deoxycorticosterone.

In the tumour tissue cultivated for 17 days, a higher percentage of cells rich in lipids and electron-dense bodies could be observed. Since by this time the hydrocortisone production of the culture decreased, in spite of continuous ACTH-treatment, it may be assumed that the lipid-rich cells are of reduced ability as regards hydrocortisone production. It is possible that the lipid-rich cells are involved in cholesterol accumulation or production of precursors, which are converted to hormone in the cells with hypertrophied AER.

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Dr. D. Szabó
Institute of Experimental Medicine
Hungarian Academy of Sciences
P.O.B. 67
H-1450 Budapest, Hungary