the secretory granules in the zymogen cells differentiated at the same stage. Using the light microscope, no cells such as the chief cells (pepsine secreting cell) can be seen in 19–20-day-old rat embryos, and of course no secretory granules of the chief cells. Before the morphological differentiation of the chief cells, sometimes many secretory granule-like granules can be observed in the apical part or in the supranuclear portion of the epithelial cells (where it coincides with the mucous part of the surface epithelial cells of adult rats). However, the author has no evidence to prove that these granules may be the peptic granules. With the light microscope, it is difficult to relate the peptic activity with the morphological source.

Zusammenfassung. Lysin, Histidin, Arginin, Tyrosin und Isoleucin wurde schwangeren Ratten täglich injiziert. In den von den injizierten Tieren geborenen Ratten tritt die Sekretgranula in den Hauptzellen ca. 2 Wochen früher auf als in den Kontrollen.

T. NISHIOKA[5]

2nd Department of Anatomy, Wakayama Medical University, 9 Kyuban-cho, Wakayama 640 (Japan),
24 July 1973.

[5] Acknowledgments. I wish to thank Prof. K. FUJIE for consistent help and kind directions.

The Effect of Diphtheria Toxin on Primary Cell Cultures of the Adult Human Liver

The human liver is seriously damaged during diphtheria and, under this pathological condition, cloudy swelling, cytoplasmic vacuolation, fatty degeneration and, finally, necrosis of the hepatocytes occur. Moreover, diphtheria toxin induces considerable mitochondrial swelling in guinea-pig hepatocytes, followed by lesions which are very similar to those observed in the human liver[1,2].

Nevertheless, it is very difficult to ascribe some of these effects to the toxin per se rather than to post-mortal degeneration and to remarkable circulatory modifications causing anoxia.

The effect of diphtheria toxin was also studied on the human liver cell cultures of the Chang's strain, which constitute a biological substrate free from hormonic, nervous, circulatory and immunological influences. At the concentration of 0.005 MLD/ml diphtheria toxin causes, in these cell cultures, cytoplasmic vacuolation, fatty degeneration, disappearance of mitosis and death of the cells[3]. Unfortunately, the Chang's strain is a very dedifferentiated cell line, which has lost most of liver cells morphological and biochemical characteristics[4]. The cytopathic effect obtained in these cells by treatment with diphtheria toxin is, therefore, quite similar to that observed in other cell strains derived from human and animal tissues different from the liver[5,6].

Primary adult human liver cell cultures were successfully obtained not long ago[7] but actually they can be considered as a suitable experimental substrate which demonstrate the morphological features that are characteristic of liver parenchimal cells. In this paper we have studied the effect of different concentrations of purified diphtheria toxin on human hepatocytes cultured in vitro in order to gather further information on the relationships between diphtheria and hepatic lesions in the man.

Materials and methods. Cell cultures. Adult human liver cell cultures were prepared by the method of ZUCKERMAN et al.[8]. Liver tissue was obtained by surgical biopsies from patients hospitalized for peptic ulcer. Liver cells were grown in Eagle's medium (Difco) containing 50 µg/ml of cephaloridine and supplemented with 20% of fetal calf serum (Microbiological Associates) on polythene discs placed into glass cups containing 1.0 ml of nutrient medium and incubated at 35 °C in air atmosphere enriched with 5% CO_2. After about 2 weeks, a suitable layer of cells was obtained and the cultures were then utilized for the experiments.

Reagents. Highly purified diphtheria toxin (kindly supplied by the Eli Lilly, Indianapolis, U.S.A.) lot. 00098, containing 600 Lf/ml was used. This toxin was diluted in the nutrient medium to obtain a final concentration of 0.1 and 1.0 Lf/ml respectively. The final pH was 7.3. Anatoxin was obtained in our laboratory from this same toxin by routine procedure. Antitoxin (horse serum, supplied by the Sclavo Institute, Siena, Italy) containing 500 AU/ml was used.

Experiments. The nutrient medium was eliminated from the glass cups and substituted with 1.0 ml of toxin dilutions. The cell cultures were then placed in a 35 °C incubator. After 3, 6, 12, 24 and 48 h the cultures were extracted from each glass cup, twice washed with Eagle's medium at +4 °C, fixed and stained as reported elsewhere[9]. These specimens were examined under a Leitz Orthoplan phase-contrast microscope. Fluorescence microscopy was carried out by fixing some specimens in ethanol for 10 min and staining with acridine orange (Merck) 1:2000 in Michaelis buffer, pH 3.5. A number of preparations were also stained by Herxheimer's method to demonstrate

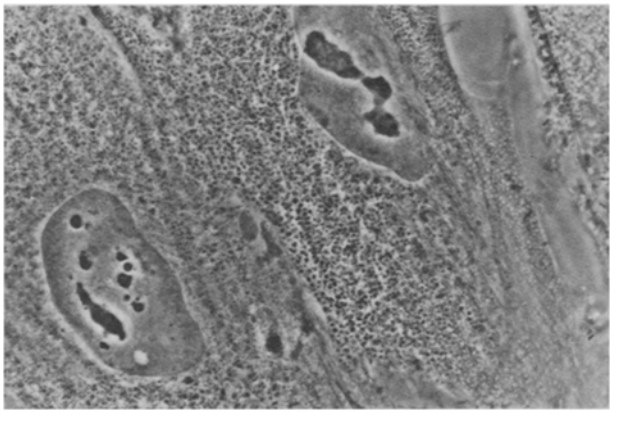

Fig. 1. Two normal liver cells in culture. Note the large nuclei and the numerous round mitochondria scattered in the cytoplasm.

[1] A. FONNESU and C. SEVERI, Biophys. biochem. Cytol. *2*, 293 (1956).
[2] D. NEUBERT and H.J. MERKER, Proc. Pharmac. Meeting, Prague 1963.
[3] G. PENSO and G. VICARI, Rendic. Ist. sup. Sanità *20*, 655 (1957).
[4] J.L. BRESLOW, H.R. SLOAN, V.I. FERRANS, J.L. ANDERSON and R.I. LEVY, Expl. Cell Res. *78*, 441 (1973).
[5] E.S. LENNOX and A.S. KAPLAN, Proc. Soc. exp. Biol. Med. *95*, 700 (1957).
[6] C. PLACIDO-SOUSA and D.G. EVANS, Br. J. exp. Path. *38*, 664 (1957).
[7] F. FULTON, J. gen. Microbiol. *22*, 416 (1960).
[8] A.J. ZUCKERMAN and F. FULTON, Br. J. exp. Path. *48*, 20 (1967).
[9] F. PARADISI, Experientia *22*, 373 (1966).

lipids. Controls were carried out by substituting plain nutrient medium or anatoxin at the same concentration of diphtheria toxin or, finally, antitoxin-toxin mixture in neutralizing ratio for diphtheria toxin dilutions.

Results. Our results demonstrate that diphtheria toxin induces a remarkable cytopathic effect in human hepatocytes cultured in vitro. Following contact with the highest dose of toxin, the hepatocytes lose their normal appearance within 3 h while a high degree of fatty degeneration is present after 12 h. As the dose of toxin is reduced, some differences in appearance of the cytopathic effect were observed: with the lowest dose of toxin, in fact, after 3 h of incubation a moderate degree of vacuolar degeneration and some fat drops in the cytoplasm can be observed, while the most important morphological changes occur after 24–48 h of incubation. In cell cultures treated with both concentrations of toxin, vacuoles containing inclusion bodies appear in the cytoplasm of the hepatocytes. These vacuoles are scattered in the cytoplasm are 1–2 in number per cell, are roughly spherical and optically inhomogeneous and range in size from 1.5 to 4.0 μm (Figure 3). The dense bodies contained within the vacuoles are not stained with the Sudan IV and are not fluorescent in the specimens stained with acridine orange. As for their size and general characteristics, these vacuoles may be regarded as pinocytotic vesicles which have engulfed foreign material.

Diphtheria toxin does not induce any modification in the volume or size of the mitochondria of these cells (Figure 2). In the latest phase of intoxication, the mitochondria almost completely disappear as numerous vacuoles of different size become visible in the cytoplasm (Figure 3). The observations carried out by fluorescence microscopy exhibit a rapid decay of brick-red cytoplasmic fluorescence which diminished in intensity with the simultaneous appearance of cytoplasmic vacuolation, and nearly disappears after 12 h, using the lowest toxin concentration, and after 3–6 h using the highest.

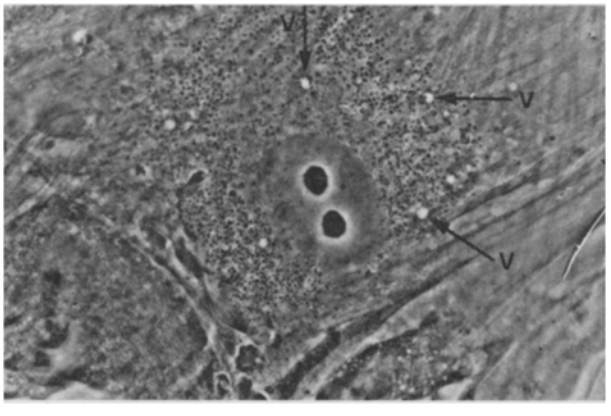

Fig. 2. Liver cell culture treated with 0.1 Lf/ml of diphtheria toxin for 3 h. Some vacuoles appear in the cytoplasm (arrows).

Fig. 3. Liver cell culture treated with 0.1 Lf/ml of diphtheria toxin for 12 h. Some inclusion bodies (IB) appear in the cytoplasm containing several vacuoles (V).

Discussion. These results show that the primary cell cultures from adult human liver are highly susceptible to diphtheria toxin which induces in these cells about the same morphological changes (cytoplasmic vacuolation and fatty degeneration) which are characteristic of liver cells of subjects who died from diphtheria, and of liver cells of diphtheria toxin-intoxicated guinea-pigs. Cytoplasmic vacuolation can, then, be due to the action of diphtheria toxin per se and not only to the anoxia or to post-mortal phenomena. Fatty degeneration is a characteristic cytoplasmic damage induced by diphtheria toxin, correlated to the inhibition of protein synthesis caused by toxin[10,11].

The uptake of toxin by hepatocytes occurs probably by pinocytosis, mainly through the large vesicles containing dense bodies which rapidly appear in their cytoplasm. Similar incorporation of different proteins into hepatocytes has been described in man in some pathological[12] and experimental conditions[13,14].

Our results indicate further that in hepatocytes cultured in vitro diphtheria toxin penetrates into the cells by an active uptake mechanism, very similar to that of the cells which posses as specific pinocytotic activity, as guinea-pig leukocytes[15]. Finally, diphtheria toxin induces remarkable mitochondrial swelling in hepatocytes of intoxicated guinea-pigs and also in different kinds of cell cultures[1,2,16]. On the contrary, any mitochondrial change appears in hepatocyte cultures in our experimental conditions. We are unable, at present, to explain this difference in the response of hepatocytes in vivo and in vitro.

Riassunto. Gli Autori hanno studiato l'effetto della tossina difterica su colture primarie di cellule di fegato umano adulto. La tossina, assunta probabilmente per pinocitosi, determina negli epatociti alterazioni assai simili a quelle presenti nelle cellule epatiche di soggetti deceduti per difterite e nel fegato della cavia intossicata con tossina difterica. Essa non determina invece rigonfiamento mitocondriale quale è stato rilevato in altri tipi di colture cellulari nelle stesse condizioni sperimentali.

F. Paradisi, A. Trapani[17] and L. Graziano[18]

Instituto di Clinica Medica, Seconda Facoltà di Medicina, Nuovo Policlinico, Via S. Pansini, I-80131 Napoli (Italy), 18 February 1974.

[10] N. Strauss and E.D. Endee, J. exp. Med. *109*, 145 (1959).
[11] I. Kato and A.M. Pappenheimer, J. exp. Med. *112*, 329 (1960).
[12] H.G. Hers, Biochem. J. *86*, 11 (1963).
[13] G. Hug and W.K. Schubert, J. Cell Biol. *35*, C1 (1967).
[14] A.G. Morell, R.A. Irvine, I. Sternlieb and I.H. Scheinberg, J. biol. Chem. *243*, 155 (1968).
[15] I. Mesrobeanu, C. Bona, L. Ioanid and L. Mesrobeanu, Expl. Cell Res. *42*, 490 (1966).
[16] F. Paradisi, Pathologia Microbiol. *30*, 481 (1967).
[17] Head surgeon, Cardarelli Hospital, Napoli (Italy).
[18] Acknowledgment. We are indebted to Mr. R. Gentile for his excellent technical collaboration and to Dr. G. F. Puccini of the Eli Lilly Italia for the generous supply of diphtheria toxin.