Ultrastructural Alterations Caused by Cytostatic Sugar-Alcohol Derivatives

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SUMMARY

Comparative examinations have been made regarding changes in Shay chloroma cells caused by three sugar-alcohol derivatives, namely dimethanesulphonylmannitol, dibromomannitol, and dibromoduleitol, its stereoisomer.

The ultrastructural alterations caused by these agents were found to be similar, the only differences between their respective effects being their duration and the time at which the regenerative changes appeared. Dibromoduleitol had the most lasting effect. While large doses of the three drugs elicited sudden grave nuclear changes, the administration of small doses was followed by grave cytoplasmic changes which lasted several days; signs of significant nuclear damage were not registered. Changes in mitochondrial size, due to the action of the said substances, have been quantitatively determined. It was a few hours after the administration of mannitolmyleran and dibromomannitol and 24 hr after that of dibromoduleitol that a rise in the number of lysosomes, their congestive enlargement, and the appearance of an increasing number of autophagic vacuoles were observed. These changes dominated the picture during several days following the treatment, a phenomenon indicative of a persistent and intensive action of the autophagic mechanism. Regarding the effect of the examined drugs on tumors, the significance of the activation of the lysosomal apparatus and that of autophagic processes are emphasized.

The study deals further with the diverse forms of mitotic disturbances caused by sugar-alcohol derivatives and with the disturbed regeneration of the nuclear membrane as manifested by the appearance of quadrilamellar membranes whose structure has also been studied.

INTRODUCTION

Production of less toxic and more selective cytostatic compounds is a fundamental purpose of cancer chemotherapy. This concept led to the production and the chemotherapeutic application of new cytostatic drugs such as 1,6-dimethanesulphonylo-α-mannitol, known as mannitolmyleran (MM) (33, 89, 92) and of brominated sugar-alcohols including, besides several other halogenic sugar-alcohol derivatives, 1,6-dibromo-1,6-dideoxy-α-mannitol, known as dibromomannitol (DBM) (17, 18, 39, 40). Its stereoisomer, 1,6-dibromo-1,6-dideoxy-dulcitol, known as dibromoduleitol (DBD), was synthesized and subjected to toxicologic and pharmacologic tests (36, 41, 46, 47).

Pharmacologic examinations and oncologic experiments proved these drugs to be considerably myelotoxic, and their antitumor effect was demonstrated on various tumors, tissue cultures, and leukemic animals (16-19, 33, 44-47, 65, 67, 76). Mannitolmyleran and dibromomannitol are now extensively used for the treatment of chronic myeloid leukemia (24, 27, 28, 78, 81-83). The clinical testing of dibromoduleitol also gave favorable results (24, 25).

All of the three examined compounds are structurally similar, being alpha-omega substituted hexitol. Their biologic action shows fundamental similarity in that all of them inhibit the myeloid system. Recent investigations have nevertheless revealed certain differences in respect to their chemical and physicochemical properties, as well as certain aspects of their biologic action (25, 26, 36, 38, 41, 46, 47). The drugs in question also differ in solubility (38, 41). Another difference between dimethanesulphonyl esters and bromine derivatives is that mannitolmyleran is stereo-specifically bound to the α-mannitol configuration, whereas in the case of dibromated compounds the stereoisomer of dibromomannitol, i.e., dibromoduleitol, is likewise efficient (18, 35, 36, 41, 46, 47). Regarding biologic action, the hematologic effect of MM is not as electively myelotoxic as, for instance, that of DBM (14-17, 18, 29). The effect of DBD on the blood picture differs from that of DBM inasmuch as the former is more lymphotrophic (66). It has been demonstrated by Kellner and Németh (44, 46, 47, 66) that DBD has a particularly broad antitumor spectrum and that it inhibits the growth of neoplasms which fail to respond to the other two compounds. DBD has, moreover, a more delayed action. Despite such differences in respect to biologic action, the general effect of all three drugs, manifested by a strong and elective myelopoietic inhibition, is essentially similar. The mode of action of the compounds under review is still far from being fully explored, and experiments with different methods have been, and are still being, carried out to elucidate this problem (6, 34, 54, 55). The present study was designed to study the effect of the three compounds on tumor...
cells by electron microscopic examinations. Considering the myelotrophic action of the drugs, Shay chloroma of myeloid origin was chosen as test object.

MATERIALS AND METHODS

Ten- to fourteen-day-old rats of the Chester Beatty strain (CB) were intraperitoneally inoculated by Németh's method (64) with saline cell suspension prepared from solid tumor nodes of the Shay chloroma. Two to three litters, i.e., 25 animals, were used in each experiment for the study of every drug to be examined, and the experiments were repeated one to three times. As a rule, treatment was started on the 6th to 8th day following inoculation. A single intraperitoneal dose was administered in the majority of cases, while the drugs were introduced perorally or subcutaneously in certain cases. Too, experiments were made to observe changes caused by the repeated administration of smaller doses (divided dosage). Table 1 shows the LD₅₀ of the examined compounds as well as the doses applied in the present study.

We studied the effect of the drugs on samples collected 1, 3, 6, 9, 12, 24, 48, 96, 120, 144, and occasionally 168 hr after the treatment.

The young animals, intraperitoneally inoculated with Shay tumor, were killed by cervical dislocation; the test material, taken from the solid tumorous infiltrate of the open abdominal cavity, was immediately placed in a 2 percent solution of osmium tetroxide buffered according to Palade's method (74). Fixation at 4°C usually lasted one hr. Dehydration was effected in increasing concentrations of alcohol. Having treated the material with absolute alcohol, we transferred it to propylene oxide, then to a mixture of propylene oxide and araldite; this done, it was embedded in araldite. An ultramicrotome, type LKB, served for the preparation of sections. As a rule, double staining was applied: we treated the sections for one hr with a fresh mixture composed of the saturated aqueous solution of uranyl acetate and a 96 percent solution of alcohol; the sections were contrasted for 20 min according to the method of Reynolds (80). Some sections, reserved for the demonstration of acid phosphatase activity, were not counterstained with lead. Photomicrographs were taken with an electron microscope, type JEM-6C.

The technic of Miller and Palade (63) was employed for the acid phosphatase reaction. Postfixation with osmium tetroxide and contrasting with lead were dispensed with in these cases.

RESULTS

It is not proposed to expatiate here upon the ultrastructural features of untreated Shay chloroma cells, as they were described in detail in a previous communication (58).

A single large dose of the examined drugs caused grave damage to the Shay chloroleukemic tissues in a very short time. The electron density of the nuclei decreased considerably; the chromatin substance formed scattered rough, lumpy clusters in the nucleoplasm, along the nuclear membrane, and around the nucleolus (Fig. 1). No significant changes in nucleolar organization were observed. The cytoplasmic organelles showed signs of slight damage at this stage. The administration of large doses was followed by rapid extensive cell destruction after 24 hr; apart from deteriorating cellular organelles the cytoplasm was filled with lipid droplets and autophagic vacuoles containing various disintegrating organelles. Most of the autophagic vacuoles contained myelin figures pointing to an advanced degradation of the cell constituents. The cell membrane of many cells appeared to be ruptured, and the intercellular spaces were inundated by deteriorating cell organelles and cellular detritus. Also, separate myelin elements of lamellar structure were seen in the intercellular spaces.

Since the rapid rate of cell destruction following the administration of large doses made it impossible to follow chronologically the damages suffered by the cell organelles, subsequent experiments were carried out with four times smaller doses in each examined drug. In still other experiments, the small

Table 1

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<tr>
<th>Commercial name</th>
<th>Agent used</th>
<th>Rat LD₅₀ (mg/kg)</th>
<th>Dose (mg/kg) and route</th>
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<tr>
<td>Mannitolmyleran (MM)</td>
<td>1,6-Dimethanesulphonoxy-a-mannitol</td>
<td>2900 i.p.</td>
<td>2000 i.p. 500 i.p.</td>
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<td>Dibromomannitol (DBM)</td>
<td>1,6-Dibromo-1,6-dideoxy-a-mannitol</td>
<td>1600 i.p.</td>
<td>1000 i.p. 250 i.p. 600 p.o. 300 p.o. 300 s.e. 80 i.p.a</td>
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<tr>
<td>Dibromodulcitol (DBD)</td>
<td>1,6-Dibromo-1,6-dideoxyc dulcitol</td>
<td>470 i.p.</td>
<td>250 i.p. 60 i.p. 200 p.o. 100 s.e. 20 i.p.a</td>
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The doses and LD₅₀ of the agents applied.

* Given 3 times.
doses were divided into three fractions administered on three successive days.

The observed changes were similar no matter whether the drugs were introduced intraperitoneally, perorally, or subcutaneously, and irrespective of whether the small dose was administered as a single dose or in fractions. In order to avoid repetitions the observed changes are not grouped chronologically but according to organelles.

Mitochondrial lesions appeared early and remained predominant after the administration of all examined drugs. A moderate swelling of the mitochondria and a decrease in the electron density of their matrix were observed between the 3rd and the 12th hr following treatment. These phenomena appeared 3 to 6 hr after treatment with MM and 6 to 12 hr after the administration of DBD. One or two days after the treatment, swollen, rounded mitochondria with electronlucent matrix were found in the majority of cells, and the cristae in them seemed to be more or less damaged, fragmented, and detached from the limiting membrane, while the double limiting membrane usually showed no impairment. Such changes were already conspicuous 12 hr after treatment with MM, whereas they became pronounced only 24 hr after the administration of dibromohexitol. Also, the double limiting membrane of some mitochondria appeared to be damaged, fragmented, and discontinuous. Mitochondrial changes were the same after divided dosage (Fig. 2). Seeing that the swelling of the mitochondria appeared early and was marked after the administration of all examined sugar-alcohol derivatives, we carried out measurements with a view to determining the extent of distensions. We determined the length of both the longitudinal and the transverse diameter of 300 mitochondria in treated and untreated tumors. Average values are listed in Table 2.

Since the values seemed to show approximately normal distribution, we determined the statistically significant differences of each treatment with reference to the control values. Computations of this kind were made according to Goldstein's methods (30) (see Acknowledgments). Values obtained by means of Student's t-test are presented in Table 3. Since the critical value of $t$ was 1.96 at the $P = 0.05$ level of confidence, increase in the mean values of the diameters was significant in all cases.

Mitochondrial changes were accompanied by a massive accumulation of lipid droplets in the cytoplasm (Fig. 2).

Another striking phenomenon elicited by the treatment was a series of lysosomal alterations manifested among others by the increased number of lysosome-like corpuscles. The number of pigment granules of the dense-core type showed a simultaneous diminution so that only occasional such granules were observed in the tumor cells some time after the treatment. Lysosome-like corpuscles contained in Shay's chloroleukemic tumor cells are electron-dense oval structures with diameters varying from 0.3 to 1.0 $\mu$. Their bulk was encountered near the nucleus in the Golgi region and the adjacent areas (Fig. 3). A positive acid-phosphatase reaction proved their lysosomal character. Lysosomal accumulation became marked 12 hr after treatment with MM or DBM, and somewhat later, i.e., after 24 hr, in the case of DBD. Only mitochondrial alterations preceded the increase in the number of lysosomes, a phenomenon indicating that, apart from mild mitochondrial changes, the appearance of lysosomes was not only early but occurred in tumor cells with almost unimpaired subcellular organization. The next observed phenomenon was a considerable enlargement of the lysosomes (with diameters between 1 and 3 $\mu$) and their increasing inhomogeneity (Fig. 4). This was accompanied by the appearance of smaller and larger autophagic vacuoles in an increasing number of tumor cells at the stage of interphase. Not only their number but also their size was found to increase with advancing time (Fig. 5). Autophagic vacuoles were surrounded by double or multiple membranes, while segregated cytoplasmic components, rough-surfaced endoplasmic reticular fragments, ribosomes, lipid droplets, and parts of deteriorating membranes were observed in their cavity (Fig. 6). Again, cellular components were no longer recognizable in other vacuoles whose cavity was filled with an amorphous or finely granular material and sometimes with rough lumps of electron-dense substance. Many autophagic vacuoles contained smaller or larger myelin structures. Such figures were frequently observed also outside the vacuoles as separate structures in the cytoplasm of damaged cells. Intensive acid phosphatase activity was demonstrable at the ultrastructural level in the majority of the autophagic vacuoles (Figs. 7, 8). The number of large congestive lysosomes and autophagic vacuoles reached a peak 48 to 72 hr after the treatment. Along with the appearance of signs pointing to a process of regeneration, i.e., at 96 to 168 hr, the number of lysosomes and autophagic vacuoles began to diminish. Occasional vacuoles were nevertheless encountered also during the period of regeneration.

Increase in the number of lysosome-like structures was especially marked after the repeated administration of divided doses: the number of autophagic vacuoles and myelin figures was considerably larger than after single doses. In addition to structures and lesions as described in the foregoing, the cytoplasm of impaired tumor cells often contained vacuoles including virus-like particles provided with electron-dense nucleoid and an outer membrane (Figs. 5, 15, 16). The average

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<th>Table 2</th>
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<tr>
<td>Diameter (A)</td>
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<tr>
<td>Longitudinal</td>
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<tr>
<td>Control tumor cells</td>
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<tr>
<td>After treatment with DBD</td>
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<td>After treatment with DBM</td>
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<td>After treatment with MM</td>
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Average values of longitudinal and transverse diameters of mitochondria in treated and untreated tumors. See Table 1 for abbreviations.

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<tr>
<td>Longitudinal</td>
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<tr>
<td>After treatment with DBD</td>
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<td>After treatment with DBM</td>
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<td>After treatment with MM</td>
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Student's "$t$" values of the average longitudinal and transverse diameters of mitochondria following treatment. See Table 1 for abbreviations.
diameter of these particles, like that of particles observed in untreated Shay tumor cells, measured 900 Å. High-resolution photomicrographs (Fig. 17) proved the limiting membranes of both the vacuoles and the virus-like particles to be unit membranes. Virus-like particles were encountered also in the autophagic vacuoles of treated tumor cells and in the dilated intercellular spaces (Figs. 15, 16). The ultrastructure of these particles was identical with that of particles described from untreated Shay tumor cells.

Simultaneously with the increasing number of lysosomes the amount of the rough-surfaced endoplasmic reticulum was found to have increased. As in the case of the lysosomes, here, too, there was a certain chronologic difference in the effects of the examined compounds. Increase in the amount of endoplasmic reticulum was pronounced 12 hr after treatment with MM or DBM only after 24 hr following the administration of DBD. The increased amount of the rough-surfaced endoplasmic reticulum persisted and was observed also in cells containing autophagic vacuoles (Fig. 6). The free ribosomes aggregated to small clusters in the first hours after treatment with MM or DBM and only 24 hr after the administration of DBD (Figs. 3, 6). The cells became increasingly poor in free ribosomes with advancing cytoplasmic impairment.

The Golgi zone remained unchanged in most of the treated tumor cells. The Golgi components of cells containing lysosomes and autophagic vacuoles often showed strong acid-phosphatase activity (Figs. 7, 8).

In contrast to grave nuclear damage caused by large doses, cells which were in interphase showed no early nuclear changes after treatment with small doses. During the interphase an increasing number of tumor cells contained strongly lobated and dented nuclei 24 to 48 hr after the treatment (Fig. 13).

It is worthy of note that nuclei and nucleoli with comparably unimpaired structure were found in cells whose gravely damaged cytoplasm was engorged with autophagic vacuoles.

An electron-dense, amorphous, and sometimes fibrillar substance was found to have accumulated in the intercellular spaces of the treated tumors (Fig. 15).

Not only cells which were in the interphase but also dividing cells were affected by the examined drugs. It was especially by treatment with DBD that cell division seemed to have become pathologic. We observed phenomena that pointed to a disturbance of both mitotic and amitotic cell division. Mitotic cell division was frequently accompanied by a disturbance in the reorganization of the nuclear envelope.

The nuclear substance developed into a highly dense, bizarrely tortuous mass. Only certain short portions of its surface were covered with a nuclear envelope-like double membrane, while a peculiar quadrilamellar membrane was observed at some distance from the nucleus (Fig. 12). The bulk of the nuclear substance formed a highly dense, uniform mass in other cases, while part of the chromatin formed a separate structure at some distance from the developing nucleus. Too, tumor cells were observed in which part of the nuclear substance had developed into a regular nucleus in the course of division, while another part formed chromosome-like clusters in the vicinity of the regular nucleus without any sign pointing to the formation of a nuclear envelope (Fig. 9).

Changes indicating a disturbance in the reorganization of the nuclear envelope were frequently observed also in tumor cells that, having passed the phase of division, were at the stage of interphase. Certain parts of the envelope covering the surface of the finally developed nuclei consisted of not two but four or more layers (Fig. 10), while, in other cases, it was in the cytoplasm, i.e., independently of the nucleus, that longer or shorter segments of the quadrilamellar membranes were seen (Fig. 11). While the inner pair of membranes had a smooth surface, ribosomes appeared to be attached to the outer membranes.

The number of damaged tumor cells was found to diminish with advancing time. Signs of regeneration became increasingly numerous. Accordingly, degenerating cells were found side by side with regenerated, apparently intact ones. The quota of damaged tumor cells diminished in the later course of the experiments, giving place to cells similar to those seen in untreated tumors. Regenerated cells, however, were more abundant in rough-surfaced endoplasmic reticulum than the control tumor cells. It occurred principally in the form of narrow cisternae, although vesicular forms, too, could be seen.

With advancing time, an increasing number of multinuclear giant cells appeared among the population of regenerating tumor cells (Fig. 14). They contained nuclei of varying sizes mostly of medium electron density. The chromatin substance was chiefly concentrated along the nuclear membrane, and the cytoplasm was rich in free ribosomes, part of which, constituting rosette-like clusters, appeared in the form of polyosomes. Both intact and impaired mitochondria were observed. The amount of rough-surfaced endoplasmic reticulum was small in the cytoplasm of the multinuclear giant cells and vesicular elements were predominant in the Golgi zone. The cytoplasm contained lipid droplets, while pigment granules of the dense-core type were rare. Presenting a fairly intact ultrastructure, the giant cells were often found to have separated out of the cell population. Many of them developed microvilli on their surface. Loosening of the interconnection between the cells is, however, no special characteristic of giant cells. Dilated intercellular spaces occurred also between regenerating mononuclear tumor cells.

The observed changes characteristic of the period of regeneration appeared at different times following treatment with various examined compounds. Signs of regeneration appeared 72 hr after treatment with DBM, and the tumorous tissue was found to have markedly regenerated at 96 hr. Cytoplasmic damage was still very pronounced at this time after treatment with DBD, and signs of regeneration did not appear until the 144th or 168th hr after the treatment. Signs of regeneration were observed 96 hr after the administration of MM, and it was at 120 hr that a population of regenerated tumor cells could be observed.

DISCUSSION

The first noteworthy phenomenon observed in the present experiments was the fact that large doses of all examined compounds elicited considerable damage within a few hours in the majority of tumor cells. We further observed a conglu-
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tination of the chromatin substance into electron-dense clusters and its “lumpy disintegration,” a phenomenon described by Kellner et al. (46, 47) and by Sugar (85) from the Guérin carcinoma of rats treated with large doses (400 mg/kg of DBD). Nuclear damages caused by large doses were followed in the case of Shay’s carcinoma by such rapid cell necrosis (usually accompanied by karyorrhexis and pyknosis) that it was not possible to follow in detail cellular alterations caused by the drugs under review. We applied, therefore, four times smaller doses in subsequent experiments.

Having witnessed the extremely grave nuclear damages following administration of large doses, it was surprising that after treatment with small doses, cytoplasmic damages rather than nuclear dominated the picture.

Ultrastructural changes were fundamentally similar after treatment with any of the examined drugs, whereas, as has been noted in the foregoing, there were qualitative differences between the effects of large and small doses. Theoretically, there are numerous possibilities in the interaction between chemotherapeutic agents and cellular constituents. It has emerged from the present investigations that, among other things, such interaction depends on the dosage which is operative in a given case.

The present experiments have confirmed, on the ultrastructural level, the fact that there exists a difference in the toxicologic properties of the three examined agents. The existence of such a difference has been proved by biologic methods (19, 45–47); it also manifests itself in the biochemical effects of the agents (34). Using DBM, only about half as much as the MM dose was required to achieve the same effect, while with DBD even a quarter of the DBM dose was sufficient. While not proposing to expatiate here on details regarding differences between the respective effects of the three agents (they will be discussed in a later part of this paper), it should be noted that most of the cells had regenerated by 96 or 120 hr following treatment with MM and DBM, while changes indicating grave cellular damage still dominated the picture at this time after the administration of DBD; it took 144 to 168 hr for the first signs of regeneration to appear after treatment with DBD. As for persistence of ultrastructural alterations, DBD was found to have the most delayed action, a phenomenon in harmony with the experimental results obtained by Kellner et al. (46, 47). Mitochondrial swelling proved to be an early and regular change produced by small doses of the sugar-alcohol derivatives, which change was later accompanied by serious alterations of the mitochondrial structure. Also, mitochondrial dysfunction supervened early, manifested by the accumulation of lipid droplets simultaneously with the swelling of the mitochondria. It is commonly known that the reaction of mitochondria to various agents is by no means a specific one (51). Our observations in this respect are nevertheless worthy of note, since they present the first data showing that a damage of mitochondria, which play a central role in the energy metabolism of cells, has to be taken into account when estimating the effect of sugar-alcohol derivatives.

Lysosomal alterations were the most characteristic changes produced by small doses of sugar-alcohol derivatives in Shay’s tumor cells. An increase in the number of lysosomes was registered after 24 hr, no matter which of the examined drugs had been administered and irrespective of the manner of administration. It was later followed by a considerable enlargement of the lysosomes and the appearance of an increasing number of autophagic vacuoles. These phenomena dominated the picture for several days. They were still more pronounced after the administration of divided doses. Besides an increase in the number of lysosomes, the aggregation of ribosomes and an increase in the amount of rough-surfaced endoplasmic reticulum were also observed. It should be noted that it was in cells with fairly intact subcellular organization that the lysosomes began to increase in number soon after the treatment, and that further cell damages supervened only later.

The early increase in the number of lysosomes, accompanied by an increase in the amount of rough-surfaced endoplasmic reticulum, the congestive enlargement of the lysosomes, the appearance of great masses of autophagic vacuoles, and the chronologic succession of these phenomena seems to prove that one was dealing with a series of changes which indicated the activation of lysosomes and the stopping of autophagic processes induced by the administered compounds.

In earlier communications we described a more or less pronounced increase in the number of lysosomes following treatment with various agents (5, 10, 52, 54, 56, 59). The appearance of lysosomes and autophagic vacuoles in such masses, seen after a treatment with the examined sugar-alcohol derivatives, has never been observed by us in connection with other drugs.

Lysosomes appearing in tumor cells following treatment with sugar-alcohol derivatives are both numerous and, as a rule, extremely large, a phenomenon that has been described as "congestive enlargement" of the lysosomes (19, 21, 23). Aggravation of the congestion resulting in the rupture of lysosomes may then lead to cellular disorganization and cytolysis. A mechanism may become operative in the cells even without a rupture of lysosomes, one that enables them to secrete and digest circumscribed portions of their own cytoplasm (23, 31, 73). Various terms have been coined for this process of cellular autophagia (21, 23, 31, 73). Autophagia may be especially pronounced in cells which undergo cytomorphosis in connection with differentiation or induced changes (21, 23). It is, according to de Duve (22), possible that pathologic autophagia is a more important factor in cytolysis than true lysosomal rupture.

A highly important problem in connection with the increase in the number of lysosomes, with their congestion and with the formation of autophagic vacuoles, is the significance of these phenomena for the fate of cells. Examinations made at different times concerning the chronology of the process are a great facility in trying to ascertain its true nature. We always followed this practice when studying the effect of chemotherapeutic agents; it enabled us to form a fairly true picture regarding the nature of autophagia arising after the administration of sugar-alcohol derivatives.

We assumed that the appearance of autophagic vacuoles in otherwise intact cells may be a morphologic manifestation of reparative processes. In the later course of the experiments,
we observed, however, a quantitative increase and enlargement of autophagic vacuoles. Their sum total occupied an increasing portion of the cells, and this phenomenon continued for several days, while an increasing number of digestive residua and myelin structures was accumulating in the vacuoles. It was proved by the observed phenomena that the mechanism of autophagia was insufficient for counteracting the toxic stimuli and for eliminating the consequent noxious products, the heavy accumulation of which resulted in grave damage and even in cell necrosis. It is only in this way that the extensive destruction of cells accompanied by the appearance of vast numbers of macrophages, a phenomenon especially pronounced after treatment with divided doses, is amenable to interpretation. We are, therefore, justified in stating that the phenomenon of induced autophagia constitutes an important factor in the effect of sugar-alcohol derivative on tumorous growths. The question arises here as to what factors are responsible for the intensification of autophagia and how it can be reconciled with existing notions and theories concerning the action of the examined drugs. It is, therefore, necessary to determine the cytostatic properties of these compounds and to ascertain those which are involved in the activation of lysosomes and the formation of autophagic vacuoles.

We have to consider those factors in the action of the compounds which may be involved in lysosomal activation. The compounds at issue may react with the nucleophilic groups of the macromolecules or may undergo hydrolysis. Protons, chloride, bromide, or methanesulphonate ions may arise in both cases. Potent intracellular acids such as methionic acid or bromhydric acid may develop during the reactions of dimethanesulphonyl and dibromohexitol (36, 41).

Intensive release of acids may, despite the known effective buffer mechanism of the cells and the organism, lead (at least temporarily) to local changes of pH in the cell organelles, among others in the lysosomes and their vicinity, so that lysosomal enzymes may reach the optimal value of pH. Local modifications of the pH value and a displacement of ionic equilibrium, occurring in the immediate vicinity of the macromolecular system by which cellular organelles are built up, may change the condition and function of the macromolecules and the permeability of intracellular membranes. These alterations may give rise to a swelling of the mitochondria and are also of paramount importance concerning the mechanism of these agents because they offer optimal conditions for the activity of lysosomal enzymes. Focal intracellular autophagic processes may thus be started, autophagic mechanisms may become operative, or autophagy may strongly intensify processes manifested morphologically by the observed ultrastructural changes caused by the examined compounds.

Lysosomal alterations, induced by sugar-alcohol derivatives, are regarded by us as important, because no such phenomena have been registered in connection with any other examined compound and also because they are, in our opinion, the chief characteristic of the action of the drugs at issue.

Being aware of the significant role played by the lysosomes in intracellular, catabolic processes (6, 23, 71, 72) and of the theory advanced by de Duve (20) according to which the lysosomes may be utilized for the intensification of chemo-

therapeutic potency and for obtaining increased selectivity, it is not surprising that this subject has come into the foreground of interest. In recent years numerous workers have investigated the role of lysosomes in the action of antitumor agents (1, 5, 7, 8, 20, 59, 68-70, 84, 86). These communications are in agreement with our observations and tend to show that the phenomenon in question is a truly important factor of the cytostatic effect. There are also attempts (7, 8) to combine cytostatic therapy with the administration of vitamin A known as the labilizer of lysosomal membranes, in order to promote the participation of lysosomal enzymes in the destruction of cells and so potentiate the antitumor action of the drug. Combinations of this kind promise especially favorable results in connection with drugs which predominantly have properties like those of the sugar-alcohol derivatives examined in this study.

According to current evidence, segregation of cytoplasmic components is the first step in the formation of autophagic vacuoles, and the appearance of demonstrable hydrolyses in the vacuolar cavity is the second one (20-23, 72, 73). Opinions are divided as to the manner in which enzymes accumulate in autophagic vacuoles (22, 23, 37, 87). Granules of the dense-core type, a special form of lysosome [storage granules (93), pigment granules (58)] contained in Shay's chloroma cells, were observed to become regularly fused with the developing autophagic vacuoles. Following treatment, both the storage granules (Fig. 5) and typical lysosomes (Fig. 3) did appear in the cytoplasm. The fusion of these typical lysosomes, as well as that of the Golgi vesicles, was also seen with the segregated areas of the cytoplasm.

Smaller or larger myelin structures were frequently found in autophagic vacuoles and sometimes in the cytoplasm as well. These structures are supposed to arise in the course of the decomposition of lipoproteins which form the membranes of cells (23, 87) and are believed to be the result of the dissociation of phospholipids and proteins.

It has been noted that treatment with sugar-alcohol derivatives induces various disturbances of cell division. The presence of quadrilamellar membranes in tumor cells was frequently observed. Considering their submicroscopic structure we regard these membranes, in agreement with Buck (12), who described similar elements from Walker 256 carcinosarcomas and termed them spindle lamellae, as nuclear membranes preformed in the course of abnormal cell division caused by the drugs under review, so that the appearance of quadrilamellar membranes seems to be indicative of a disturbed regeneration of the nuclear envelope.

We observed the appearance of multinuclear giant cells in the period of regeneration following treatment with sugar-alcohol derivatives. The formation of multinuclear cells has been described also after treatment with X-irradiation (4, 50), and with different mustard-nitrogen derivatives and other chemotherapeutic preparations (2, 3, 13, 32, 42, 48-50, 53, 60). Multinuclear giant cells, induced by MM and DBD, have been described by Kellner et al. (45-47) on the basis of light microscopic examinations. Several authors pointed out that the origin of giant cells, formed under the effect of chemotherapy agents, is far from being uniform (11, 42, 61, 62),

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since a fusion of cells, fragmentation of nuclei, disturbed, abortive mitosis, and amitotic cell division may all be involved in their genesis (11, 43, 75, 77, 90). It is therefore probable that multinuclear cells, arising after treatment with sugar-alcohol derivatives, too, originate in different ways.

Regarding the efficacy of chemotherapeutic drugs, the appearance of multinuclear giant cells is by no means a negligible phenomenon, and it would therefore be extremely useful to obtain further data regarding their biologic value. Opinions are widely different in this respect (11, 42, 43, 79).

Concerning the multinuclear cells formed under the effect of sugar-alcohol derivatives, we established that in the great majority of these cells no ultrastructural alterations pointing to degeneration or imminent cell-death could be seen.

Morphologic analysis alone, however, does not suffice for estimating the biologic value of the cells.

Cellular damages caused by the sugar-alcohol derivatives were seen accompanied by the accumulation of a highly electron-dense, amorphous substance in the intercellular spaces. The nature of this substance is not cleared up yet.

It is worthy of note that, especially after the administration of DMB and DBD, we frequently observed a great number of virus-like corpuscles which were often found in the lysosomes and the autophagic vacuoles. The factors, presumably involved in this particular phenomenon, were analyzed in a previous communication (57, 58).

We think the above data regarding the antitumor effect of sugar-alcohol derivatives, obtained by electronmicroscopic examinations, have shed light on numerous hitherto hidden aspects in connection with the action of these compounds.

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REFERENCES


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Fig. 1. Low-resolution photomicrograph of Shay's chloroleukemic tissue six hr after intraperitoneal treatment with a 250 mg/kg dose of dibromomannitol. The electron density of the nucleus (N) is considerably diminished, and the chromatin substance (ch) is accumulated along the nuclear envelope (Nem). The nucleolus (Nu) shows normal structure and is surrounded by a large amount of associated chromatin (ach). The narrow cytoplasmic rim of the cells is rich in free ribosomes; low-resolution photomicrograph reveals no significant alteration of the cytoplasmic organelles. The space in the center of the picture seems to be taken up by a highly swollen damaged cell, containing no nucleus—only cell detritus. X 9,000.

Fig. 2. This picture illustrates mitochondrial alterations (M), induced by the examined drugs. The mitochondria are swollen, and their electronlucent matrix contains damaged, fragmented cristae and numerous intramitochondrial granules. Also the limiting membrane of the mitochondria is damaged at certain points. Numerous lipid droplets (Li) can frequently be seen in the cytoplasm near the damaged mitochondria. The cytoplasm contains slightly increased, rough-surfaced, endoplasmic reticulum (rER) and typical and atypical pigment granules (pg). The treatment has not produced essential change in the ultrastructure of the nucleus (N). Electron photomicrograph taken 48 hr after treatment with divided doses (20 mg/kg, 3 times) i.p. of dibromomannitol. X 20,000.

Fig. 3. Beside the damaged mitochondria (M), a great number of lysosomes (ly) and occasional pigment granules (pg) can be seen in the Golgi zone (G), with the cross section of a centriole (C) between them. The cytoplasm contains, besides rough-surfaced endoplasmic reticulum (rER), a great number of free ribosomes (r). Most ribosomes are aggregated in the form of rosette-like polysomes (pr). Electron photomicrograph taken 24 hr after treatment with divided doses (20 mg/kg, 3 times) i.p. of dibromomannitol. X 24,000.

Fig. 4. In addition to an increase in the number of lysosomes (ly), a part of them is considerably enlarged (lysosomal congestion) and their substance can be seen to have become heterogeneous. Among the congestive lysosomes, numerous lipid droplets (Li) and damaged mitochondria (M) and myelin containing small autophagic vacuoles (aV) could be seen. Electron photomicrograph taken 48 hr following treatment with divided doses (20 mg/kg, 3 times) i.p. of dibromomannitol. X 22,000.

Fig. 5. Following treatment, an increasing number of small autophagic vacuoles (aV) are formed in the Golgi zone (G) between numerous single-membrane bound granules of the dense-core type (storage granules) (pg). Note swollen mitochondria (M) and a row of pinocytotic vesicles (pV) on the cell surface. The ultrastructure of the nucleus (N) and the nucleolus (Nu) is unchanged. Virus-like particles (vP) can be seen in the intercellular compartment. Electron photomicrograph taken 48 hr after intraperitoneal treatment with divided doses (80 mg/kg, 3 times) i.p. of dibromomannitol. X 15,000.

Fig. 6. In addition to several autophagic vacuoles (aV) some of which also contain myelin figures (m), comparatively unimpaired mitochondria are visible in the cytoplasm of the tumor cells. The increased amount of rough-surfaced endoplasmic reticulum (rER) has a lamellar structure, while the free ribosomes are aggregated to small clusters (pr). The nucleus is of medium electron density, and the chromatin (ch) is evenly distributed. Electron photomicrograph taken 48 hr after treatment with a single dose (250 mg/kg) i.p. of dibromomannitol. X 18,000.

Fig. 7. Acid phosphatase activity in Shay's chloroleukemic-cell. A small amount of lead phosphate, the end product of the reaction, is located in the Golgi zone (G), while a large amount is also visible in the area of lysosomes (ly) and autophagic vacuoles (aV), which are abundant in the tumor cells at this time, a phenomenon pointing to intensive acid phosphatase activity. Electron photomicrograph taken 24 hr after treatment with divided doses (80 mg/kg, 3 times) i.p. of dibromomannitol. X 27,000.

Fig. 8. Granular lead phosphate, pointing to intensive acid phosphatase activity, can be seen in the cavity of differently sized lysosomes (ly) and autophagic vacuoles (aV) with which the cytoplasm is packed. It is worthy of note that the vacuoles containing myelin structures (which may be regarded as residual corpuscles) are usually devoid of lead phosphate. Electron photomicrograph taken 72 hr after treatment with a single dose (250 mg/kg) of dibromomannitol. X 10,000.

Fig. 9. In the course of disturbed mitosis, the development of a nucleus with perfectly regular structure (N), as well as a large amount of nuclear substance (ch), can be seen to form chromosome-like clusters in the cytoplasm. No nuclear envelope is visible on the surface of the aggregated nuclear substance. Note Golgi apparatus (G), centriole (C) near the nucleus, and dilated mitochondria (M) between the piles of chromosomes. Electron photomicrograph taken 48 hr after treatment with divided doses (20 mg/kg, 3 times) of dibromomannitol. X 27,000.

Fig. 10. Circumscribed portion of the nuclear surface covered by a quadrilamellar membrane (Qm) continuous with the outer membrane of the nuclear envelope. The free end of the latter extends to the Golgi zone (G) of the cytoplasm. Dilatation of the perinuclear space (arrow), is observable in the said portion of the nuclear surface. Electron photomicrograph taken 24 hr after treatment with divided doses (80 mg/kg, 3 times) i.p. of dibromomannitol. X 35,000.

Fig. 11. Advanced mitotic disturbance, due to the treatment, manifests itself also by the existence of longer or shorter portions of the quadrilamellar membranes (Qm) in cells during interphase. The nuclear surface of these cells is often lobated (N). Electron photomicrograph taken 24 hr after treatment with divided doses (80 mg/kg, 3 times) i.p. of dibromomannitol. X 35,000.

Fig. 12. Detail of dividing Shay chloroma cell. The chromosomes are coalesced to form a continuous, bizarre, tortuous mass of chromatin (ch). Certain parts of its surface are covered by a double membrane (arrow) indicating the formation of nuclear membrane. Organelles of the cytoplasm are arranged around the chromatin substance at the periphery of the cell. The minute portion of a quadrilamellar membrane (Qm) is visible near the periphery. Besides mitochondria (M), the cytoplasm contains scattered pigment granules (pg). Electron photomicrograph taken 48 hr after treatment with a single dose (250 mg/kg) of dibromomannitol. X 12,000.

Fig. 13. Shay's chloroleukemic tumorous tissue obtained from rat 48 hr after having been intraperitoneally treated with a 500 mg/kg dose of mannitolmyleran. The picture shows a tumor cell with a bizarrely lobated nucleus (N) of unusual shape. The nuclear substance is of medium electron density, with the chromatin (ch) evenly distributed. An increasing number of tumor cells, containing such irregular nuclei, have been encountered after a treatment with all examined drugs. Mitochondrial (M) changes and a multiplication of congestive lysosomes (ly) can be seen in the cytoplasm. Electron photomicrograph taken 48 hr after treatment with a single dose (500 mg/kg) of mannitolmyleran. X 14,000.
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Fig. 14. Multinuclear tumor cell taken from the abdominal cavity of Shay chloroleukemic rat, 120 hr after treatment with a single dose (500 mg/kg) of mannitolmyleran. The cell shows four nuclei (N) or nuclear lobes of different sizes in cross section. The nuclei are of medium electron density; part of the chromatin substance (ch) is arranged along the nuclear membrane. The cytoplasm contains an abundance of evenly distributed free ribosomes (r). The structure of the mitochondria (M) is well preserved; their matrix is sometimes somewhat more electronlucent. Propigment granules (ppg) are to be found in the narrow Golgi zone (G). Occasional microvillus-like (Mt) cytoplasmic processes are observable on the surface of the rounded cell. × 30,000.

Fig. 15. Detail of seriously damaged Shay chloroma cell 72 hr after treatment with a single dose (500 mg/kg) of mannitolmyleran. The cytoplasm is filled with two large vacuoles and lipid droplets (La). One of the vacuoles contains myelin figures (my), the other smaller one (Va) contains virus-like corpuscles (vP) with electron-dense, ovoid nucleoids of 800 to 900 Å size. The nuclear surface is lobated (N), and irregular aggregates of highly electron-dense amorphous substance (arrow) can be seen in the distended intercellular space. Virus-like particles (vP) occur in the intercellular space as well. × 28,000.

Fig. 16. An increasing number of virus-like particles (vP) with electron-dense nucleoids has been observed; they were found in the cytoplasmic vacuoles and the dilated intercellular space of the tumor cells of treated animals. Electron photomicrograph taken 24 hr after intraperitoneal treatment with divided doses (80 mg/kg, 3 times) of dibromomannitol. × 35,000.

Fig. 17. High-resolution photomicrograph reveals that the virus-like (vP) corpuscles contain horseshoe-shaped, electron-dense nucleoids and are provided with limiting membrane of the unit-membrane type. Electron photomicrograph taken 24 hr after treatment with divided doses (80 mg/kg, 3 times) of dibromomannitol. × 170,000.
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