Ultrastructural Pancreatic Beta-Cell Changes in Rabbits after Small and Large Doses of Alloxan


SUMMARY

The effect of a single injection of alloxan monohydrate, varying in dosages from 12.5 to 200 mg./kg. body weight, on the ultrastructure of pancreatic β cells was investigated in 100 adult rabbits killed five minutes to fourteen days after alloxan administration.

Large doses of alloxan (50 to 200 mg./kg.) produced severe hyperglycemia due to marked β-cell damage characterized by translucent areas in the hyaloplasm, mitochondrial swelling, disintegration of the intercellular membrane, and nuclear pyknosis, followed by the dissolution of β-cell granules and, ultimately, disintegration of the cell. The sequence in which these changes occur suggests that alloxan, in high dosages, exerts its principal damaging action, at least initially, upon the paramembranous portion of the β-cell cytoplasm.

After small doses of alloxan, 12.5 or 25 mg./kg., hyperglycemia failed to occur. In these animals there was early degranulation, distension of the cisternae of the endoplasmic reticulum, variable dilatation of the profiles of the Golgi apparatus, and formation of morphologically atypical β-cell granules.

Small doses of alloxan, while not destroying the pancreatic β cells, alter the β-cell secretory apparatus sufficiently to induce subdiabetes, so that a normally nondiabetogenic dose of cortisone (1 mg./kg.) converts this state into one of overt diabetes. The ultrastructural and functional pancreatic β-cell changes after small doses of alloxan alone are quite similar to those induced by large doses of alloxan in cortisone-pretreated rabbits.

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Since the discovery of selective pancreatic β-cell toxicity of alloxan by Dunn, Sheehan and McLetchie1 in 1943, the light-microscopic features of alloxan-induced β-cell damage have been described repeatedly.2-4 In general, clumping of aldehyde fuchsin positive β-cell granules, shrinkage of the β-cell cytoplasm, nuclear pyknosis and loss of intercellular cohesion were noted within the first few hours after alloxan administration. As the cells disintegrated, the debris remained aldehyde fuchsin-positive.5-6 At variable times after the first day, the islets were found to consist mainly or exclusively of α and δ cells and only occasional β cells; the latter were usually degranulated. In several communications from thi laboratory6-9 it was shown that pretreatment of rabbits with diabetogenic doses of cortisone protects the pancreatic β cells against the toxic effect of alloxan. In the present study the fine structure of pancreatic β cells of alloxanized rabbits was investigated. Special attention was paid to the question of whether small, nondiabetogenic doses of alloxan produced pancreatic β-cell changes comparable to those seen in cortisone-pretreated alloxanized animals.

MATERIAL AND METHODS

The study was carried out on 100 white adult New Zealand rabbits of both sexes, weighing from 2 to 4 kg. Each rabbit received a single injection of alloxan monohydrate intravenously. The dosage of alloxan varied from 12.5 mg. per kg. to 200 mg. per kg. body weight. Blood glucose determinations by the Nelson-Somogyi method10 were performed daily on most of those rabbits which were permitted to survive for more than one day, and also on some others that were not utilized for the electron microscopic study. Alloxan dosages and time intervals between the injection of alloxan and killing are shown in table 1. Four additional rabbits served as normal controls.

All animals were killed by injecting an overdose of Nembutal five minutes to fourteen days after the administration of alloxan. The pancreatic tissue of all rabbits was removed immediately after death. One portion was fixed for two hours in ice-cold, buffered 1 per cent osmic acid solution with added sucrose11 and embedded with epon 812 according to the method of Luft.12 Sections were then cut with a glass knife on...
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TABLE 1
Number of rabbits killed at various time intervals after alloxan injection

<table>
<thead>
<tr>
<th>Alloxan (mg./kg.)</th>
<th>Minutes</th>
<th>Time interval</th>
<th>Hours</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>2 2</td>
<td>2</td>
<td>4 2</td>
<td>2</td>
</tr>
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<td>2 2</td>
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<tr>
<td>25</td>
<td>3 2</td>
<td>2</td>
<td>2 4</td>
<td>2</td>
</tr>
<tr>
<td>12.5</td>
<td>2 2</td>
<td>2</td>
<td>4 2</td>
<td>2</td>
</tr>
</tbody>
</table>

The Servall Porter-Blum microtome after localization of islets in thick sections by light microscopy. Thin sections were viewed and photographed with an RCA EMU-3G electron microscope. The second portion of pancreas was placed in Zenker-formalin solution and processed as previously described. Paraffin sections, 3 µ thick, were stained with a combined periodic acid-Schiff trichrome stain and by a modification of Gomori’s aldehyde fuchsin method.

RESULTS

Blood sugar values

Of six rabbits receiving 12.5 mg. per kg. of alloxan, and of six with doses of 25 mg. per kg., none became diabetic. After the injection of 50 mg. per kg. of alloxan, five of ten rabbits displayed hyperglycemia; blood sugar values began to rise on the second or third post-allowxan day and ranged up to almost 400 mg. per 100 ml. Six rabbits with 100 mg. per kg., and eight with 200 mg. per kg. of alloxan became severely hyperglycemic from the second day of alloxan administration onward. Blood sugar figures in these two groups generally ranged from 300 to 600 mg. per 100 ml.

Light microscopy

The light microscopic findings were similar to those described previously, but differed according to whether a large or a small dose of alloxan had been given. Generally, after administration of 12.5 or 25 mg. per kg. of alloxan no distinctive alterations were noted in β cells, except for variable degranulation after five to ten minutes. In animals that had received doses ranging from 50 to 200 mg. per kg. of alloxan, there was frequently early shrinkage of β cells followed by a loss of cellular cohesion. After one or two hours the aldehyde fuchsin-positive β-cell granules had frequently lost their discrete outlines and had merged into an ill-defined aldehyde fuchsin-positive mass (compare figure 1A and figure 1B). Whereas such cells were obviously destined to perish, there were other islets, especially in animals which had received 50 mg. per kg. of alloxan, in which most or all of the β cells survived and showed few changes except for variable degranulation (figure 1C). Finally, after two or more days, many islets contained but few or no β cells; those still present appeared degranulated (figure 1D).

Electron microscopy

The cells of the pancreatic islets of untreated control rabbits showed the characteristic morphology as previously described (figure 2). In assessing the spectrum of ultrastructural changes after alloxan administration, it soon became apparent that some pancreatic β cells were heavily and irreversibly damaged, whereas in others the alterations were of a qualitatively different and nonlethal nature. In general, the severity of the observed damage paralleled the amount of alloxan administered, but exceptions to this rule were occasionally evident.

Large doses. The earliest alteration in severely affected cells, often seen only five to ten minutes after the injection of 50, 100 or 200 mg. per kg. of alloxan monohydrate, was to be found in the appearance of electron-translucent areas in portions of the β-cell cytoplasm (figures 3 and 4). Such “empty” areas were sometimes most conspicuously present near the cytoplasmic membrane or near a capillary. This hyaloplasmic change reached its maximum intensity several hours after the administration of alloxan (figure 5). Mitochondrial swelling (figures 3 and 4) and nuclear pyknosis (figures 3 to 5) were other early changes in such severely damaged cells.

One or two hours after the administration of a large dose of alloxan, the intercellular membranes began to disintegrate (figure 4) and the β cells frequently lost their cohesion with one another, similar to the picture seen by light microscopy (figure 1B). After five and more hours, intercellular membranes could often no longer be identified between adjacent β cells (figure 5).

In view of these pronounced and extensive early alterations it was noteworthy to find that in many of these severely damaged β cells the secretory granules remained unaffected for a considerable period of time (figures 3 and 4). As long as five hours after the injection of a large dose of alloxan, many granules still maintained a normal or nearly normal appearance within cells which were far advanced on their way towards necrosis (figure 5).

Disintegration of β cells reached its climax towards the end of the first and on the second day after the
administration of 50, 100 or 200 mg. per kg. of alloxan (figures 6 and 7). The envelopes of some nuclei that had failed to become pyknotic were seen to dissolve and their chromatin was noted to spill into the surrounding cytoplasm (figure 6). The contents of the secretory granules became clumped or disappeared altogether (figures 6 and 7). By the second day, macrophages containing many autophagic bodies had moved into the disintegrating β cell fragments (figure 7).

From the fourth day on, all β cells had disappeared from many of the islets which, therefore, consisted exclusively of α and δ cells (figure 8). However, in occasional islets, surviving β cells were identified (figure 9). Their endoplasmic reticulum was sometimes arranged in conspicuous, fingerprint-like arrays. They contained but few secretory granules, usually in the vicinity of the cell membrane. Often glycogen deposits were noted (figure 9).

**Small dosages.** After the administration of 12.5 or 25 mg. per kg. of alloxan the observed β-cell alterations were quite distinct from those described above. Hyaloplasmic changes, mitochondrial damage, nuclear pyknosis and, finally, necrosis of the cell were rarely, if ever, present. Instead, there was moderate degranulation visible within five to fifteen minutes after the injection of the drug (figure 10). At the same time, the profiles of the endoplasmic reticulum became distended (figure 10) and, after one or two hours, the vesicles of the Golgi apparatus were also dilated (figure 11). Four to seven hours after the administration of a small dose of alloxan, atypical granules began to appear; they varied in size, configuration and electron density of the cell membrane.
and included granules resembling a crescent (figure 12), a doughnut, or a target (figure 13). Some of these alterations, especially degranulation, the presence of occasional atypical granules, and dilatation of the cisternae of the endoplasmic reticulum, persisted until the end of the two-week observation period (figure 14).

DISCUSSION

The present study demonstrates that the ultrastructural pancreatic β-cell changes after the injection of alloxan monohydrate depend primarily upon the size of the dose administered.

A large dose, such as 100 or 200 mg. per kg. of alloxan, causes, initially, translucency of portions of the hyaloplasm, mitochondrial swelling, disintegration of the cell membrane with early loss of intercellular cohesion, and nuclear pyknosis, followed later by the dissolution of β-cell granules and, ultimately, death of the cell. Although many attempts have been made to elucidate the mechanism of action of large doses of alloxan ever since Dunn, Sheehan and McLetchie1 discovered alloxan-induced β-cell necrosis in 1943, no definitive conclusions have been reached to date.

During the last few years, Lazarow and co-workers have published a series of papers3-10 which have added new aspects to this matter. After exposing islet tissue of the toadfish—which, in this species, is topographically separate from the exocrine pancreas—to the action of radioactive alloxan-C-14 and of mannitol, they have concluded that alloxan is not selectively concentrated by islet tissue, that neither alloxan nor its decomposition products enter the islet cell, and that
alloxan appears to act exclusively and selectively on the permeability of the β-cell membrane. The manner in which alloxan alters the permeability was thought to be related to its reaction with a dithiol site in the membrane. All subsequent changes were said to be secondary to the increase in permeability of the β-cell membrane, induced by alloxan.

In our study, translucent areas of the β-cell hyaloplasm, especially in the vicinity of the cell membrane, were observed after large doses of alloxan. A coarse vacuolization of the β-cell cytoplasm, with initial preservation of secretory granules, was also noted by Falkmer and Olsson in electron microscopic studies several hours after the administration of 200 to 350 mg. per kg. of alloxan. The disintegration of the intercellular membranes, with subsequent loss of cohesion between neighboring, heavily damaged β cells, follows rather than accompanies this paramembranous hyaloplasmic clearing. This sequence of events could mean that alloxan in high dosage, exerts its initial action upon the cytoplasmic membrane of the cell. However, it is also possible and appears more likely that alloxan first attaches itself to a hyaloplasmic component and that the disintegration of the cell membrane is a secondary phenomenon. Earlier studies have implicated a reduction, by alloxan, in the availability of unbound extramitochondrial sulfhydryl groups as a major factor in the ultimate necrobiosis of the β cell.

Considerably damaged and only slightly altered δ cells were occasionally seen side by side within the same islet or, more often, in different islets of the same animal. This, as well as the varying responses of different islets of the same animal to alloxan, suggests that different islets may have different sensitivities to alloxan.
different individual rabbits to the same dose of alloxan, can possibly be explained by differences in the functional state of individual β cells at the time of alloxan administration. It is known that the effect of alloxan on the β cell is modified by a large number of exogenous and endogenous factors, including cortisone and blood glucose levels.

After small doses of alloxan, such as 12.5 or 25 mg. per kg., the observed β-cell changes are quite different from those demonstrable after the injection of 100 or 200 mg. per kg. Disintegration and necrosis of β cells are almost never seen. Instead, there is early, pronounced and often persistent degranulation, distinct early and sometimes persisting dilatation of the cisternae of the endoplasmic reticulum, and variable distension of the profiles of the Golgi apparatus. The occurrence of an early, transient increase in the number of β-cell granules, as described by other authors, was not observed in the present experiment. Obviously, these features are expressions of cellular stimulation and hyperactivity.

The ultrastructural changes in pancreatic β cells after small doses of alloxan are similar to those produced in cortisone-pretreated rabbits which were given a large dose of alloxan. Under both conditions the β cells ap-
PEARL LOSS THEIR ABILITY TO PRODUCE OR TO STORE INSULIN IN THE USUAL MANNER.

In the cortisone-pretreated, alloxanized animals it was shown that a prediabetic state exists which can be transformed into overt diabetes by additional small, normally nondiabetogenic doses of cortisone. Unpublished preliminary observations in this laboratory suggest that a prediabetic state is also present in rabbits after the injection of 12.5 or 25 mg. per kg. of alloxan. These animals do not show a raised blood sugar level, but a normally subdiabetogenic dose of 1 mg. per kg. of cortisone causes a significant hyperglycemia (figure 15). This type of response to cortisone was obtained in three of five rabbits who had received 12.5 mg. per kg. of alloxan, and in all of twenty-three rabbits after 25 mg. per kg.

FIGURE 9
Portions of several surviving B cells two weeks after the injection of 200 mg. per kg. of alloxan monohydrate. The animal was severely diabetic. The endoplasmic reticulum (E) is concentrically arranged. Disseminated glycogen deposits (G) are noted. Only occasional granules (arrows) are present along the intercellular membranes. GC = Golgi complex; M = mitochondria. X 8,250.

FIGURE 10
Portions of pancreatic B cells fifteen minutes after the administration of 25 mg. per kg. of alloxan monohydrate. Most B-cell saccules (arrows) are either empty or contain weakly electron-dense material. The cisternae of the endoplasmic reticulum (E) are dilated and frequently vesicular. M = mitochondria; A = a cells. X 15,375.

The feasibility of inducing a subdiabetic state in the rat by subthreshold doses of alloxan has been shown by Lazarow. Three of nine subdiabetic rats so treated displayed a progression of their symptoms during the following six months and became overtly diabetic. In the other six animals, glucose tolerance improved during the second year after an initial stage of impairment. The ultimate course of the subdiabetic state induced in the rabbits in our own experiments is presently being studied in this laboratory.

Recently, Lazarus, Volk and Barden described two types of secretory vacuoles in the pancreatic B cell (figure 2). The vacuoles of the first type are characterized by electron-dense central nucleoids and are thought to originate from the endoplasmic reticulum. The vacuoles of the second type contain pale staining material. Their
exact site of origin is still unknown. They are thought to represent a secretion vacuole in which the insulin secretory product is in a different physical or chemical state. It was hypothesized that it is these latter vacuoles which extrude their content into the intercellular space when an appropriate secretory stimulus acts upon the cell.

The present study suggests that small doses of alloxan may impede the capacity of the β cell to manufacture or store either of these two types of granules since several days later the majority of cytoplasmic vacuoles contain hazy electron-dense material with an atypical configuration and texture. These changes in secretion vacuoles are demonstrable prior to and in the absence of overt hyperglycemia which, by itself, may cause degranulation as it does in the few surviving β cells of rabbits after large doses of alloxan. The distension and vesiculation of the units of the endoplasmic reticulum and of the profiles of the Golgi apparatus which are often seen in such degranulated β cells may signify an attempt of the alloxan-damaged cell at compensatory, albeit not totally successful, insulin production.

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ULTRASTRUCTURAL PANCREATIC $\beta$-CELL CHANGES IN RABBITS AFTER SMALL AND LARGE DOSES OF ALLOXAN

FIGURE 13
Portion of a pancreatic $\beta$ cell (B) seven hours after the administration of 12.5 mg. per kg. of alloxan monohydrate. Atypical granules are present (arrows), some showing a doughnut or target-shaped appearance. GC = Golgi complex; E = endoplasmic reticulum; N = nucleus; A = $\alpha$ cell. X 15,375.

FIGURE 14
Portions of several pancreatic $\beta$ cells six days after the injection of 12.5 mg. per kg. of alloxan monohydrate. Persistence of atypical granules is noted (arrows). The profiles of the endoplasmic reticulum are slightly distended (E). GC = Golgi complex; M = mitochondria; N = nucleus X 10,800.

REFERENCES

SINGLE DOSE OF 
12.5 mg./Kg. OF ALLOXAN  
(RABBITS A AND C) 

1 mg./Kg. OF  
CORTISONE PER DAY  
(RABBITS B AND C) 

DAYS 

FIGURE 15
Blood sugar curves of three rabbits: (A) after a single dose of 12.5 mg. per kg. of alloxan monohydrate alone; (B) after 1 mg. per kg. of cortisone acetate intramuscularly once daily; and (C) after a single dose of 12.5 mg. per kg. of alloxan monohydrate followed two weeks later by 1 mg. per kg. of cortisone acetate intramuscularly once daily for seven days. The normally nondiabeticogenic dose of 1 mg. per kg. of cortisone acetate per day produces marked hyperglycemia in the alloxanized animal.


