DIFFICULTIES IN ASSESSING RELATION OF GLUCAGON TO ALPHA CELLS

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INTRODUCTION
Conflicting reports have appeared concerning the origin of glucagon from the pancreatic A cell, as determined by glucagon assay of pancreas from animals treated with cobalt chloride (Cavallero, 1953; Volk et al., 1954; Lazarus et al., 1954; Goldner et al., 1954; De Duve, 1953; Mayer et al., 1953). Vulksteke and De Duve (1952) found, using a quantitative method that the glucagon content of the pancreas diminishes by 60% in the cobalt treated guinea pig. On the other hand, Volk et al., (1954), using qualitative method could not find a significant difference in the glucagon content of the pancreas of cobalt treated dogs and rabbits. Curiously enough, in a recent paper, Fodden and Read (1954), reported that the glucagon content of cobalt treated rabbit pancreas was about twice that of the control animal. Study of the data reveals discrepancies between the morphologic findings in the pancreas. Failure to produce identical morphologic changes following cobalt injection may account for the different results regarding the relation of glucagon to the A cell as suggested by Fodden and Read (1954). In any case, no final correlation between glucagon and the A cell can be established by the use of cobalt chloride as the selective damaging agent of the A cell unless it consistently results in a reproducible lesion of the A cell. Our own experience in this field and a consideration of the work of others therefore seems timely.

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MATERIAL AND METHODS

A total of 92 New Zealand White rabbits, of both sexes, weighing 1200 to 4500 gm. fed ad lib, with Purina Rabbit Chow were divided into 8 groups. Six groups were treated with cobalt chloride, (Fisher, Reagent quality, Nickel 0.20%), one group of untreated rabbits used as control. All animals received the cobalt chloride, in saline as a single intravenous injection. An additional group of 12 rabbits received the cobalt chloride, (B. D. H., Analar quality, Nickel 0.012%), in distilled water as subcutaneous injections. Animals were killed by intravenous air injection. Details of the procedures are as follows: Group 1: Eighteen rabbits weighing between 1800 and 4500 gm. were used as controls. Group 2: Five rabbits weighing between 2500 and 3000 gm. received 25mg./kilo of cobalt in a 5% solution. One animal each was killed at 1/4, ½ 1, 2 and 4 hours after cobalt administration. Group 3: Thirteen rabbits weighing between 3000 and 4500 gm. received 50 mg./kilo of cobalt in a 5% solution. Two animals were found dead 3 days after the treatment, while one died 6 hours after and it was autopsied about 15 minutes after death. The remaining 10 rabbits were killed some time after cobalt administration as follows: One each after 1, 6, 12, 36, 72, 96 hours; 2 at 24 hours and 2 at 48 hours. Group 4: Ten rabbits weighing between 2900 and 3800 gm. received 50 mg./kilo of cobalt in a 5% solution. Four animals died within 6 hours after cobalt injection, the remaining rabbits were killed 24 hours after cobalt administration. Group 5: Eleven rabbits weighing between 2200 and 2900 gm.
received 50 mg./kilo of cobalt in a 9% solution. Three animals died 12 to 22 hours following treatment, the remaining rabbits were killed 24 hours after cobalt administration. Group 6: Eleven rabbits weighing between 1700 and 3000 gm. received 70 mg./kilo of cobalt in a 9% solution. Two animals died 12 to 22 hours following treatment, the remaining were killed 24 hours after cobalt administration. Group 7: Twelve rabbits weighing between 2300 and 2800 gm. received 90 mg./kilo of cobalt in a 9% solution. This dose was highly toxic and all but one animal died. It was possible to perform an autopsy between 15 and 30 minutes after death of 3 of the rabbits. The time between injection and death was as follows: One died during the injection, one after 5 hours and another after 7 hours. Tissues were not taken from the other 7 rabbits because of advanced post-mortem autolysis. One rabbit was killed 24 hours after cobalt administration. Group 8: Six rabbits weighing between 1200 and 3500 gm. received subcutaneously

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40 mg./kilo in a 4% solution, one daily injection. Two animals each were killed 24 hours after the second, fourth and eight injections respectively. Another 6 rabbits weighing between 1800 and 3500 gm. received subcutaneously 60 mg./kilo in a 6% solution, one daily injection. All 6 were killed 24 hours after seven injections. Forty-one male guinea pigs were also used. Group 1: Six guinea pigs weighing between 900 and 1200 gm. were controls. Group 2: Ten guinea pigs weighing between 400 and 1300 gm. received a reduced diet so as to diminish their weight by approximately 20% within 6 days. This approximates the weight loss of treated animals. Group 3: Twenty-five guinea pigs weighing between 400 and 1400 gm. received 20 mg./kilo of cobalt chloride (B. D. H. Analar quality) in a 1% aqueous solution. Animals received daily subcutaneous injections for 7 days and were killed 6 hours after the last injection. Twelve out of 25 guinea pigs died during the experimental period. Immediately after sacrificing the rabbits, the tail and pancreas were removed and fixed in Zenker-Formol and in Aoyama’s fluid, this latter for demonstration of the Golgi apparatus; the guinea pig pancreas was fixed in Zenker-Formol only. Tissues were processed and stained following the methods previously described Bencosme (1952). Paraffin sections were serial and 2.5 μ thick. Zenker-Formol fixed tissues were stained with the Masson’s trichrome, the Gomori’s chrome alum hematoxylin stain and the Aldehyde-Fuchsin stain Gomori (1950). Aoyama’s preparations were counterstained with the Masson’s trichrome Bencosme (1952). Because it was important to separate individual A granules, the Masson’s trichrome was modified, thus differentiating individual A granules in black. In order to stain the A granules black, the Regaud’s hematoxylin was incubated in a paraffin oven for a few days until it became black and covered with a metallic film. Hemadmin- toxylarin, ripened in this way, was used for all our Masson’s trichrome stains.

OBSERVATIONS

Rabbits: Animals that died within a few hours after intravenous injection of cobalt presented symptoms of severe respiratory distress. Those that survived, did not develop diarrhea in contrast with the animals used by Volk et al., (1953). Food intake and body weight on the other hand was markedly reduced in those which survived the largest doses of cobalt. Rabbits which received multiple subcutaneous injections of cobalt besides loosing weight also showed a tawny discoulouration of their pancreas.
Figure 1. - Pancreatic islet from a rabbit which receive 70 mg/kilo of cobalt chloride and killed 24 hrs after cobalt injection, showing numerous A cells without cytological alterations. Masson' trichrome X 900.

Figure 2. - Pancreatic islet from a pregnant rabbit autopsied ½ hr after death showing pyknotic A cells Gomori’s chrome alum hematoxilin X 900.

Figure 3. - Pancreatic islet from a normal rabbit autopsied 2 hrs after death showing pyknotic A cells. Masson’ trichrome X 900.
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Microscopic Findings. In previous work it has been shown that the islets of the rabbit pancreas contain A, B and D cells. The Golgi apparatus is distinct for each type of cell and the D cell is devoid of granules Bencosme (1955). The methods used in the present work are suitable to recognize minimal cytological alterations of any of these various islet cell types. In the present experiment, we are unable to observe in any of the rabbits injected with cobalt chloride cytological changes in the A cell (Fig. 1), as reported by other investigators, such as degranulation (Volk et al., 1953; Goldner et al., 1952; Fodden, 1953a, b; Van Campenhaut and Cornelis, 1951), cloudy swelling (Fodden, 1953a, b) necrosis (Volk et al., 1953; Goldner et al., 1952; Fodden, 1953a, b) and vacuolization of the cytoplasm (Fodden, 1953a, b; Van Campenhaut and Cornelis, 1951; Van Campenhaut et al., 1954; Avezzu and Luise, 1951).

In animals which were autopsied some time after death some time after death some A cells showed nuclear pyknosis. This alteration has been interpreted by us as post-mortem phenomenon. Special study of this point was made in 24 normal rabbits weighing between 1000 and 4500 gm. These rabbits were divided into 8 groups of 3 rabbits each. Each group was killed and autopsied. The time between death and removal of the pancreas was exactly 5, 15, 30 minutes and 1, 2, 6, 12 and 24 hours respectively for a given group of rabbits. Beginning at 15 to 30 minutes after death there occurred a progressive development of nuclear pyknosis of A cells. This change increased in severity and in the number of cells affected up to about 6 hours post-mortem. This post-mortem artefact has recurred repeatedly in other experiments in which the pancreas has been examined some time after death. In these latter experiments comparable fresh tissue was also studied and the A cells did not show pyknosis. Previous investigators have described an almost complete disappearance of A cells in cobalt treated animals (Volk et al., 1953, Goldner et al., 1952, Fodden, 1953a, b, Van Campenhaut and Cornelis, 1951). To test the possibility that there was a reduction in A cells several tests were made. This was done by examining slides from equal numbers of control and treated animals without prior knowledge of the protocol. It was impossible in these tests to separate control from treated rabbits.

Figure 4. — Pancreatic islet from a guinea pig which received 7 subcutaneous injections of cobalt chloride. The A cells are severely degranulated or vacuolated. They appear clear in the picture. Masson’s trichrome X 100.

Figure 5. — Pancreatic islet from a normal guinea pig showing well granulated A cells in black. Masson’s trichrome X 400.

Figure 6. — One area from Fig. 4 at higher magnification showing degranulated and vacuolated A cells as pale staining cells. Compare with the dark stained A cell of normal guinea pig. Masson’s trichrome X 400.
Guinea Pigs. Cobalt treated guinea pigs lost an average of 20% of their weight during the experiment. In contrast with rabbits, cobalt treated guinea pigs, invariably developed moderate to severe degranulation of alpha cells after only 4 injections as shown in the guinea pigs which died during treatment. Vacuolization of a large number of alpha cells was a prominent feature in animals with the severest lesions. The nucleus of the alpha cells were in general well preserved. B and D cells were not affected in these animals (Fig. 4, 5 and 6).

DISCUSSION

The observations of Van Campenhout et al (1954) on A cell damage produced by cobalt treatment in guinea pigs has been fully confirmed in this work. On the other hand, in the rabbit, we have been unable to produce any visible change in the A cell by the use of either a single intravenous or repeated subcutaneous injection of cobalt chloride in spite of the fact that the doses given were larger than that used by others (Volk et al., 1953; Fodden, 1953b; Avezzu and Luise, 1951), who have reported A cell damage due to cobalt treatment in the rabbit. The amount used in one group was toxic enough to kill most animals within 24 hours. Moreover, the times between cobalt administration and death, include the times which have been reported showing maximal degeneration of the A cell. The weight, sex and race of the animals was also comparable to that used by other investigators; in addition, younger and older animals, as indicated by their weights, were also used. Lack of effect on the A cell was also observed by Wrenshall in dogs treated with cobalt. Similar negative results have been observed in this laboratory in experiments now in progress on rats, dogs and chickens. Negative results similar to ours have been reported with cobalt on dogs, rabbits and rats by Creutzfeldt and Schmidt (1954). On the other hand these authors were able to damage the A cell in guinea pigs treated with cobalt.

Van Campenhout and Cornelis (1951) reported an alteration in blood sugar following a single intracardiac injection of cobalt chloride and suggested that this might be due to liberation of glucagon before destruction of the A cell, but they made no mention of histological changes in the A cells in these experiments. In spite of this Volk et al., (1954), stated that Van Campenhout had so produced changes in the A cell and that they were able to confirm his work and reported necrosis and marked reduction in the number of A cells after a single injection of cobalt chloride. Since then Van Campenhout et at. (1954), have reported that they were unable to alter the A cell with a single intracardiac injection although they were able to produce its degranulation and vacuolation but no necrosis with multiple injections.

De Duve (1953) has stated that glucagon is derived from the A cell on the basis of glucagon assay from pancreas of cobalt treated guinea pigs which presented A cell lesions identical to those described by Van Campenhout et al., (1954). On the other hand, Volk et al., (1953) reported in the rabbit lesions of a different type including necrosis and reduction in the number of A cells. On the basis of glucagon extraction of pancreas with these lesions they were of the opinion that glucagon does not originate in the A cell. Of interest is the recent work of Fodden and Read (1954) who obtained from the pancreas of cobalt treated rabbit approximately twice the amount of glucagon found in the control pancreas. These authors, however, found that treatment with synthalin A which produces A cell damage in the rabbit, similar to those induced with cobalt in the guinea pig, (Van Campenhout, et al., 1954), did abolish the glucagon content of the pancreas.
It seems probable that the different opinions regarding the relation of glucagon to A cells in cobalt treated animals are based on discrepancies of the morphological findings in such animals. How widely the observations vary on the response of the A cell to cobalt chloride is attested by a brief review of the literature. A number of investigators (Volk et al., 1953; Goldner et al., 1952; Fodden 1953a, b) have described changes following one injection while along with Van Campenhout et al. (1954) we have been unable to produce any visible change with a single injection. Others as in the present work found that lesions only follow multiple injections and then only in certain species (Van Campenhout et al., 1954; Avezzu and Luise 1951; Creutzfeldt and Schmidt 1954; Wrenshall). Furthermore the type of change reported varies also. Degranulation and vacuolation of A cells without necrosis was described by Van Campenhout et al. (1954). While others have reported the most prominent lesion as necrosis of the A cell (Volk et al. 1953; Goldner et al. 1952; Fodden, 1953a, b). In our own normal and cobalt treated rabbits we have frequently noted pyknosis and disintegration of the A cell in animals which were found dead. In each of these cases we have attributed these alterations to post-mortem change because we have never seen them in a rabbit autopsied immediately after death. In this connection it is therefore not advisable to follow the practice often encountered in which all animals of an experimental group are killed together and then autopsied seriatim. While this procedure is satisfactory for ordinary histology it is not so for cytological study of islet cells, in particular of the A cell.

This observation along with the wide disagreement concerning the effect of cobalt chloride on the A cell indicates that careful morphologic control of the pancreas with eventual agreement on the effect of cobalt chloride on the A cell must be achieved before any final conclusions can be reached regarding the relation of glucagon and the A cell on the basis of damage supposedly caused by cobalt chloride.

A further factor contributing to the different results obtained on extraction of the pancreas for glucagon may be the regional differences within the pancreas in the content of A cells which has recently been observed in our studies (Bencosme).

SUMMARY

In a study involving 92 rabbits and 41 guinea pigs, no appreciable morphological alteration could be demonstrated in the cytology of the A cell of the rabbit pancreas following a single intravenous or multiple subcutaneous injections of cobalt chloride even when lethal doses were used; nor was there any discernable decrease in the number of A cells in the cobalt treated animals. Early post-mortem changes simulating A cell necrosis were discussed. On the other hand the lesions described by Van Campenhout et al. (1954) in the A cell of the cobalt treated guinea pig were fully confirmed.

RESUME

Nous n’avons pas trouvé de changements morphologiques appréciables dans les cellules A du pancreas de lapin, chez 92 animaux, après traitement unique intraveineux ou injections multiples souscutanées de chlorure de cobalt, même a doses toxiques. Nous n’avons pas non plus constaté de diminution dans le nombre de ces cellules. D’un autre côté les lesions décrites par Van Campenhout et al. (1954), dans les cellules A du cobaye, après traitement au cobalt ont été retrouvés intégralement chez 41 animaux.
WRENSHALL, G. A.: Personal’ communications.