Sensitivity of Beta Cells to Alloxan after Inhibition by Insulin or Stimulation by Glucose

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In spite of the number of investigations carried out since the discovery of the selective cytotoxic action of alloxan on the beta cells of the islets of Langerhans,[1] the mechanism by which this agent exerts its action on the beta cells is not well understood.

Okamoto,[2] Maske et al.[3,4] support the view that alloxan exerts its diabetogenic effect, as do certain other chemical diabetogenic agents, by chelating cytoplasmic zinc of the beta cells. Lazarow[5] attributes the cytotoxic effect of alloxan to its high reactivity with sulphydryl groups and the selectivity of the compound for the beta cell to the metabolic specialization of the cell for insulin synthesis.

The prolonged administration of exogenous, long-acting insulin to rats has been shown to produce marked depletion of secretory granules,[6] loss of beta-cell zinc and probably inhibition of insulin synthesis.[7] Beta-cell granulation and zinc are restored to normal levels within six to eight days following the cessation of the injections.[7]

The administration of large amounts of glucose is also known to lead to both the depletion of granules[8] and of cytoplasmic zinc[9] in the beta cells. The process of insulin synthesis can be assumed to be accelerated under these circumstances.

In the experiments presented below, the susceptibility of the beta cells to the cytotoxic action of alloxan was investigated in insulin-inhibited and glucose-stimulated beta cells.

It was found that the presence of zinc and the state of activity of the beta cells do not appear to determine the sensitivity of the beta cells to alloxan.

The level of blood glucose at the time of alloxan injection was the important factor in determining the sensitivity.

METHODS AND PROCEDURES

Injection of insulin. Young adult male rats weighing 250 to 300 gm. were injected with progressively larger amounts of NPH insulin in the morning and Protamine Zinc Insulin late in the afternoon. The doses were increased from two to three units for NPH insulin and from three to seven units for Protamine Zinc Insulin progressively within ten to fifteen days. Control rats received saline injections. The insulin injections were stopped for half of the treated group after the thirtieth day. The hyperglycemia shown in these rats for two to three days following the cessation of insulin injections was potentiated by tube feeding twice daily with 5 gm. of finely ground chow suspended in water. The rats which continued to receive insulin injections and the control group which was injected with saline throughout were handled in the same manner, except only water was given by stomach tube. Alloxan was injected on the thirty-third day four to five hours after the last tube feeding or gastric intubation and three to five hours after the last insulin injection.

Infusions of glucose. Young adult rats, weighing 325 to 400 gm., were anaesthetized; a polyethylene tube was inserted into the jugular vein one or two days before the administration of glucose.

Continuous infusion of 40 per cent glucose for seven hours. Experiments were performed on groups of eight rats. Four rats were infused continuously with 40 per cent glucose in Locke's solution at a rate of 5 gm. of glucose per kilogram body weight per hour. Two were infused with Locke's solution and the other two with 15 per cent mannitol in Locke's solution. Alloxan was injected within two minutes following the cessation of the infusion in five of the rats. Two of the glucose-infused rats and one infused with Locke's solution were injected with alloxan one hour after the end of the infusion period.

Infusion of glucose for ten minutes. Groups of rats were infused with 40 per cent glucose, 15 per cent glucose or 15 per cent mannitol in Locke's solution at a rate of 0.3 ml. per minute for ten minutes. Alloxan injections were given within two minutes after the infusion was stopped.

Injection of alloxan. Fifty milligrams of alloxan (Eastman Kodak) per kilogram body weight in a volume of 0.6 to 0.8 ml. of acid normal saline (*p*H 3.5) was injected into the dorsal vein of the penis in two seconds

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under light-ether anesthesia. Solutions were made just prior to injection.

Estimation of the severity of diabetes. After the injection of alloxan, individually marked rats were kept in groups and injected with 1.0 unit of Protamine Zinc Insulin daily for twenty days. The dosage of insulin was decreased to 0.5 unit on the twenty-first day and the rats were then transferred to individual metabolic cages for the assessment of the severity of diabetes which became evident on the cessation of insulin administration. Thus, the severity of the diabetes was assessed after the rats had recovered from the immediate toxic effects of alloxan and had lost the excess fat which had accumulated in their fat depots during the prolonged insulin treatment. This was important considering that Steiner et al.[10] have shown that the severity of ketoacidosis and the mortality of alloxan-diabetic rats depend on the size of the fat depots. The rats were kept in individual metabolic cages for three days. Total urinary glucose was estimated daily by the quantitative method of Benedict. The daily consumption of ground chow was measured. The morning of the fourth day, after food had been withdrawn for twelve hours, the rats were killed by bleeding under ether anesthesia. Blood samples were taken for glucose estimation and the pancreases were fixed in Bouin's fluid for histological examination.

The severity of diabetes was estimated by the following three parameters: (1) daily urinary glucose per gram of ingested ground chow; (2) blood glucose level at killing, and (3) histological changes of the islets of Langerhans.

RESULTS

Diabetogenic effect of alloxan after prolonged insulin treatment. Figure 1 shows the severity of the diabetes produced in the three groups as judged by the urinary glucose and blood-glucose level at the time of killing. The histological evaluation of the degree of beta-cell destruction was an equally good criterion of the severity of diabetes and correlated well with the above parameters.

The insulin-treated group showed the same severity of diabetes as the control group. This group, at the time of the injection of alloxan, had blood glucose levels ranging from 25 to 75 mg. per 100 ml.

Inhibited beta cells, with a nearly complete loss of granules and cytoplasmic zinc, proved highly susceptible to the cytotoxic action of alloxan. In contrast, 50 per cent of the rats in the period of transient diabetes following the cessation of the insulin treatment escaped diabetes. This group at the time of the alloxan injections had blood-glucose levels between 135 and 351

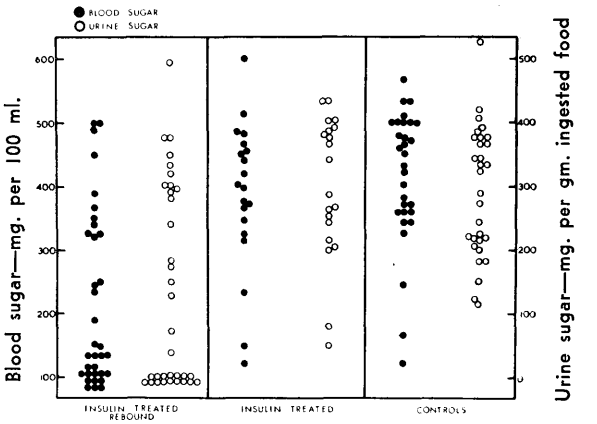

FIG. 1. Severity of diabetes twenty days after the injection of alloxan.
1. Rats treated with insulin for thirty days and injected with alloxan three days after the last injection of insulin (rebound hyperglycemia).
2. Rats treated with insulin for thirty days and injected with alloxan five hours after the last injection of insulin.
3. Control rats injected with alloxan.

mg. per 100 ml. Most of the "protected" rats had a blood-glucose level over 250 mg. per 100 ml. The beta cells of the islets two to three days after the cessation of the insulin injections are still depleted of granules and cytoplasmic zinc although in the process of recovering their ability to make insulin.[7]

Diabetogenic effect of alloxan after infusion of 40 per cent glucose for seven hours. Infusion of 40 per cent glucose for seven hours at the rate of 5 gm. per kilogram body weight per hour produces a marked loss of granules and cytoplasmic zinc of the beta cells.[11] Mannitol induces a more profound osmotic diuresis than does glucose but affects neither beta-cell zinc nor granulation.[11]

The rats injected with alloxan within two minutes following the cessation of the infusion had, at the time of the alloxan injection, blood-glucose levels ranging from 324 to 564 mg. per 100 ml. Nine out of twenty-four developed mild diabetes (figure 2). In rats alloxanized one hour after the end of the glucose infusion the blood glucose levels either had returned to or were slightly above normal at the time of the alloxan injection. Beta cells at that period are still depleted of granules and cytoplasmic zinc.[11] These rats were not "protected" at all. The mannitol-infused group was as sensitive as the control group to the diabetogenic action of alloxan, both showing the expected high incidence of severe diabetes produced in normal rats (figure 2).

Diabetogenic effect of alloxan after infusion of glucose for ten minutes. The results obtained from the

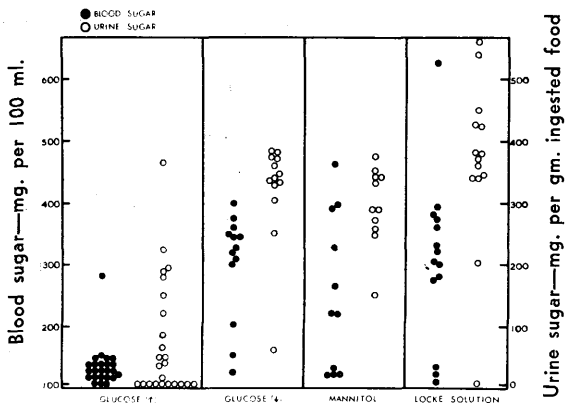

FIG. 2. Severity of diabetes twenty days after the injection of alloxan.

Glucose ↑: rats injected with alloxan within two minutes following the cessation of the glucose infusion (seven hours).

Glucose ↓: rats injected with alloxan one hour after the end of the glucose infusion (seven hours).

Mannitol: rats injected with alloxan within two minutes following the cessation of mannitol infusion (seven hours).

Locke: rats injected with alloxan within two minutes following the cessation of the infusion of Locke's solution (seven hours).

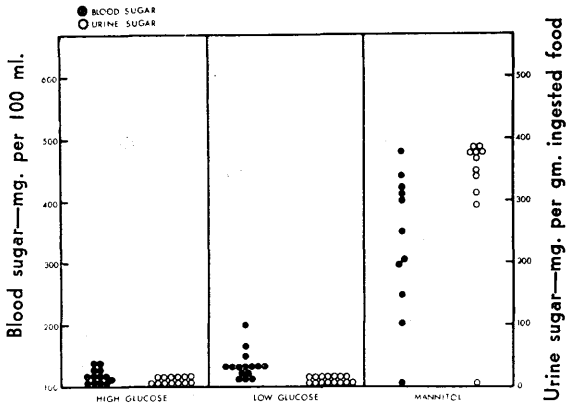

FIG. 3. Severity of diabetes twenty days after the injection of alloxan to rats having high blood glucose levels produced by a short infusion of glucose (ten minutes).

High glucose: Blood glucose 570 mg. to 1,000 mg. per 100 ml.

Low glucose: Blood glucose 280 mg. to 400 mg. per 100 ml.

Mannitol: Infused with 15 per cent mannitol.

preceding experiments suggested that a high blood-glucose level might have been the most important factor in increasing the resistance of the beta cells to alloxan. Rats infused with 40 per cent glucose showed blood glucose levels ranging from 570 to 1,000 mg. per 100 ml. at the time of the alloxan injection, and were completely "protected" against the diabetogenic action of alloxan. Only three out of the fifteen rats infused with 15 per cent glucose and having blood glucose range between 280 and 400 mg. per 100 ml. at the time of the alloxan injection developed any diabetes. The mannitol infusion again proved ineffective (figure 3).

DISCUSSION

Beta cells from which granules and cytoplasmic zinc were nearly completely depleted by prolonged administration of exogenous insulin or markedly decreased by glucose stimulation proved as susceptible to alloxan toxicity as those of normal rats. The presence of cytoplasmic zinc, therefore, does not contribute to the susceptibility of the beta cells to alloxan. The results with the insulin-treated rats also demonstrated that beta cells whose synthetic activities were inhibited were fully sensitive to alloxan.

The sensitivity of beta cells to alloxan following an infusion of glucose for seven hours depended on whether alloxan was injected immediately or one hour after the end of the infusion. Rats which were injected immediately following the infusion (blood glucose over 325 mg. per 100 ml.) were "protected." Rats which were injected one hour following the cessation of the infusion, when blood glucose was returning towards normal (below 180 mg. per 100 ml.), developed the same severity of diabetes as the control rats.

Degranulation and loss of beta-cell zinc were equally marked in both groups. The glucose level and not its sequelae (degranulation or loss of zinc) was the determining factor of the susceptibility of beta cells to alloxan.

The effect of high glucose levels in protecting beta cells from the cytotoxic effect of alloxan is not a new observation. It was first suggested by Carrasco-Formiguera and Escobar[12] and directly demonstrated by Sen and Bhattacharya.[13] Using short-term glucose infusion (ten minutes) and our standardized procedure in assessing the severity of diabetes, the protective effect of a high-glucose level per se at the time of the injection of alloxan was confirmed. The exact mechanisms by which glucose exerts this striking effect are not known.[14,15] If it could be shown that the half life of alloxan in the extracellular fluid is not significantly decreased in animals with a high glucose level, then metabolic changes in the beta cell could be postulated. In a recent paper, Volk and Lazarus[16] reported that cortisone pretreatment protected the beta cells of the rabbit against alloxan. As a possible explanation, they suggested an increased activity of the shunt pathway for glucose oxidation in the beta cell due to hyperglycemia. This would lead to an increased rate of formation of reduced triphosphopyridine nucleotide and reduced glutathione, and consequently to a more rapid

inactivation of alloxan. Such a mechanism could well be operating in the rats with high blood-glucose levels produced by the infusion of glucose.

SUMMARY

(1) Inactive beta cells depleted of granules and zinc by prolonged treatment with insulin were as susceptible to the cytotoxic effect of alloxan as beta cells of untreated rats.

(2) Rats were infused with a concentrated glucose solution for seven hours. The stimulated beta cells showed a marked loss of granules and zinc for several hours following the infusion. Alloxan was ineffective in inducing diabetes when injected immediately after the cessation of the infusion (blood glucose over 325 mg. per 100 ml.). The same dose of alloxan was fully effective when injected one hour later (blood sugars less than 180 mg. per 100 ml.).

(3) Rats were also protected against the diabetogenic effect of alloxan: (a) during the phase of post-insulin hyperglycemia, and (b) following short infusion (ten minutes) of concentrated glucose solutions.

(4) Thus the sensitivity of the beta cell to alloxan is determined by the prevailing blood-glucose level. The zinc content, the degree of granulation and the activity of the beta cells appear to have no modifying effect on the toxicity of alloxan.

SUMMARIÓ IN INTERLINGUA

Le Sensibilitate del Cellulas Beta pro le Action de Alloxano post Inhibition per Insulina o Stimulation per Glucosa

1. Inactive cellulas beta depletionate de granulos e de zinc per un prolongate tractamento con insulina esseva tanto sensibile pro le effecto cytotoxic de alloxano como le cellulas beta de non-tractate rattos.

2. Un gruppo de rattos recipeva infusiones de un concentrate solution de glucosa durante septe horas. Le stimulate cellulas beta monstrava un marcate perdita de granulos e de zinc durante plure horas post le infusion. Alloxano esseva inefficace in le induction de diabete quando illo esseva injicite immediatemente post le suspension del infusion (quando le glucosa del sanguine amontava a plus que 325 mg per 100 ml). Le mesme dose de alloxano esseva plenmente efficace quando illo esseva injicite un hora plus tarde (quando le glucosa del sanguine amontava a minus que 180 mg per 100 ml).

3. Rattos se provava etiam protegite contra le effecto diabetogene de alloxano (a) durante le phase de hyperglycemia post insulina e (b) post breve infusiones (durante 10 minutas) de concentrate solutiones de glucosa.

Assi le sensibilitate del cellula beta pro alloxano es determinate per le existente nivello de glucosa sanguinee. Le contento de zinc, le grado de granulation, e le activitate del cellulas beta pare haber un effecto modificatori super le toxicitate de alloxano.

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REFERENCES

1 Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B.: Necrosis of islets of Langerhans produced experimentally. Lancet *1*:484-87, 1943.

2 Okamoto, K.: Production of experimental diabetes mellitus and zinc reaction of islets of Langerhans. Hyogo. J. Med. Sci., Japan *1*:77-88, 1951.

3 Maske, H., Wolff, H., and Stampfl, B.: Über die Verhinderung der diabetogenen Alloxanwirkung durch vorhergehende Glucosegaben. Klin. Wschr. *31*:79-81, 1953.

4 Maske, H., Stampfl, B., and Gahn, H. 4: Untersuchungen zur Verhinderung der diabetogenen Alloxanwirkung durch vorhergegebenes Adrenalin. Z. Klin. Med. *152*:68-72, 1953.

5 Lazarow, A.: Alloxan diabetes and the mechanism of beta cell damage by chemical agents. In Experimental Diabetes, a Symposium, Oxford, Blackwell Scient. Publ., 1954, p. 49-81.

6 Latta, J. S., and Harvey, H. T.: Changes in the islets of Langerhans of the albino rat induced by insulin administration. Anat. Rec. *82*:281-96, 1942.

7 Logothetopoulos, J., Kraicer, J., and Best, C. H.: Granulation and reactive zinc in the cells of the islets of Langerhans. Effect of prolonged insulin treatment. Diabetes *10*: 367-74, 1961.

8 Gomori, G., Friedman, N. B., and Caldwell, D. W.: Beta cell changes in guinea pig pancreas in relation to blood sugar level. Proc. Soc. Exp. Biol. Med. *41*:567-70, 1939.

9 Wolff, H. P., Ringleb, D., and Amann, R.: Histochemische Untersuchungen uber das Inselzink; histophotometorische Messungen, Z. Ges. Exper. Med. *126*:390-416, 1955.

10 Steiner, D. F., Rauda, V., and Williams, R. H.: Severe ketoacidosis in the alloxan diabetic rat. Endocrinology *68*: 809-17, 1961.

11 Logothetopoulos, J.: Unpublished experiments.

12 Carrasco-Formiguera R., and Escobar, I.: Influence of a previous injection of epinephrine upon the diabetogenic effect of alloxan in rabbits. Amer. J. Physiol. *152*:609-14, 1948.

13 Sen, P. B., and Bhattacharya, G.: Protection against alloxan diabetes by glucose. Indian J. Physiol. and Allied Sci. *6*:112-14, 1952.

14 Bhattacharya, G.: On the protection against alloxan diabetes by hexoses. Science *120*:841-43, 1954.

15 Villar-Palasi, C., Carballido, A., Sols, A., and Arteta, J. L.; Sensitivity of pancreas hexokinase towards alloxan and its modification by glucose. Nature *180*:387-88, 1957.

16 Volk, B. W., and Lazarus, S. S.: Protection by cortisone pretreatment against alloxan diabetes. Arch. Path. 73:363-70, 1962.