Chlorozocin

A Diabetogenic Analogue of Streptozocin with Dissimilar Mechanisms of Action on Pancreatic Beta Cells

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SUMMARY

Chlorozotocin (chlorozocin, CLZ), the 2-chloroethyl analogue of streptozocin (STZ), was evaluated in three species of rodents. The drug is currently being used in phase II chemotherapeutic trials in man, and appears to be effective in the treatment of certain tumors. In our studies, hyperglycemia was induced in hamsters as early as 2 days after a single intraperitoneal (i.p.) injection of 30–60 mg/kg and was most striking at 4 days. Greater concentrations of CLZ (≥50 mg/kg) were required to produce hyperglycemia in CD-1 mice. Degranulation and necrosis of beta cells developed in hamsters and mice, whereas alpha and acinar cells of the pancreas revealed no morphologic changes. Hyperglycemia was not induced in rats at any concentration tested; however, animals showed abnormal carbohydrate tolerance after administration of 100 mg/kg CLZ (LD_{50} dosage).

The nature of damage by CLZ to beta cells was investigated both in vivo and in vitro. Pretreatment of hamsters with nicotinamide (500 mg/kg, i.p.) failed to alter the extent of CLZ-induced beta cell injury and associated hyperglycemia, but decreased the amount of beta cell necrosis and hyperglycemia in animals receiving STZ. The nonmetabolizable sugar, 3-O-methylglucose (3-O-MG), and 3-aminobenzamide, an inhibitor of the nuclear enzyme, polyADPribose synthetase, prevented STZ-associated damage to beta cells in islet cell cultures, but only 3-O-MG reduced CLZ-induced toxicity. Thus, in comparison to STZ, CLZ appears to be a diabetogenic agent with different species specificity and alternative mechanisms of cytotoxicity. The glucose moiety of both drugs appears critical in the induction of beta cell damage. DIABETES 1985; 34:602–10.

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Received for publication 4 July 1984 and in revised form 16 November 1984.

Streptozocin (STZ), a nitrosourea linked to position C_2 of D-glucose, exhibits selective toxicity for beta cells and is diabetogenic in laboratory animals (reviewed in ref. 1). It is unclear why STZ shows a specificity for the beta cell, although the presence of the glucose moiety is thought to be important. Several experimental observations support this statement. For example, STZ-induced diabetogenicity is ameliorated when 3-O-methylglucose (3-O-MG) or 2-deoxyglucose, nonmetabolized analogues of glucose, are administered to rodents.[2,3] Large amounts of glucose also prevent hyperglycemia and necrosis of beta cells after injection into rats of either α- or β-anomers of STZ.[3] When injected into rodents at equivalent concentrations, STZ is found in insular tissue at 3.8–fold higher amounts than methylnitrosourea (MNU), the non-glucose-containing form of STZ.[4] These data suggest that the structural glucose renders N-nitroso compounds with a unique specificity for beta cells.

This study was undertaken to determine whether another glucose-containing N-nitroso compound, chlorozocin (CLZ), a drug used in human cancer chemotherapy,[5] was diabetogenic in rodents (Figure 1). Moreover, we were interested in determining possible mechanisms of action of CLZ. Data presented here indicate that the interaction of CLZ with the DNA of the beta cell differs markedly from that observed with the classical diabetogenic agent, STZ.

MATERIALS AND METHODS

Experimental protocol to evaluate the diabetogenicity of CLZ. Male golden Syrian hamsters (Bioresearch Institute, Cambridge, Massachusetts), CD-1 mice, and Sprague Dawley rats (both from Canadian Breeding Laboratories, St. Constant, Quebec) were used at 6–8 wk of age. Rodents (N = 7–12 per treatment group) having free access to food and water were weighed before i.p. injection of CLZ (NSC #178248, the gift of Dr. Robert R. Engle, Developmental Ther-

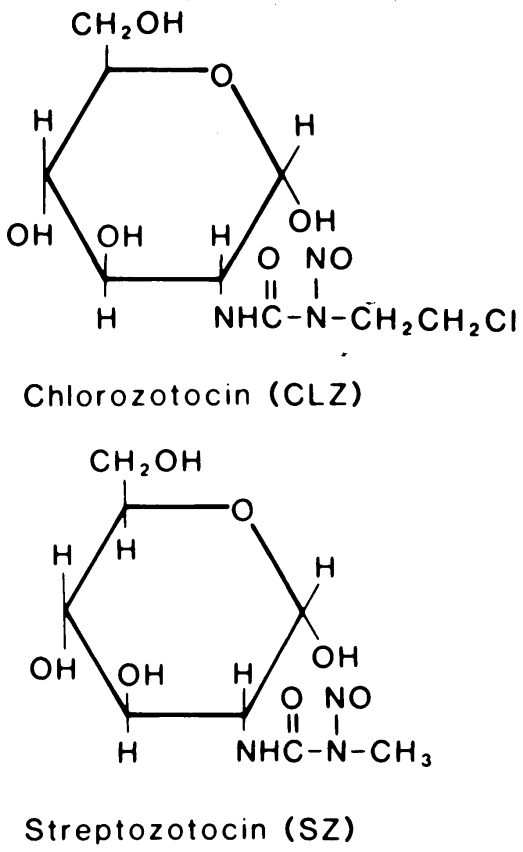

FIGURE 1. Structural formulas of chlorozocin (CLZ) and streptozocin (STZ).

apeutics Program, Division of Cancer Treatment of the National Cancer Institute; 20–100 mg/kg) dissolved in a final volume of 0.1 ml dimethyl sulfoxide (DMSO). Control animals received 0.1 ml DMSO alone. In comparative studies, hamsters were administered nicotinamide (Sigma Chemical Co., St. Louis, Missouri; 500 mg/kg)[6] i.p. in 0.8% NaCl, pH 7.4, 10 min before injection of either CLZ (40 mg/kg) or STZ (obtained from Dr. W. E. Dulin, Upjohn Research Laboratories, Kalamazoo, Michigan; 100 mg/kg, dissolved in 0.1 M citrate buffer, pH 4.4). Solutions for injection into animals were monitored spectrophotometrically at 232 nm to determine the stability of CLZ and STZ in DMSO and citrate buffer, respectively.

All animals were bled at 2, 4, and 7 days after injection. In hamsters and mice, nonfasting blood glucose concentrations were determined on samples of whole blood from the orbital sinus. Because nonfasting blood glucose concentrations were not elevated in rats after injection of CLZ (20–100 mg/kg), glucose tolerance tests also were performed on animals of this species. Rats were fasted overnight (12 h) at 4 days after injection of CLZ and a blood sample obtained before i.p. injection in double-distilled H_2O (0.1 ml) of 2 mg/g glucose. Blood glucose then was determined at 30, 60, and 120 min.

Histopathology. Randomized groups of rodents (N = 4–6) were killed on the second, fourth, and seventh days of the experiments. Other animals were killed either when moribund or after 4 wk. The liver, heart, kidneys, lung, gallbladder, and pancreas were fixed in Bouin's solution and 5 μm histologic sections were stained with hematoxylin and eosin (H and E). The pancreatic tissue was also examined by the peroxidase-antiperoxidase technique to evaluate insulin and glucagon in islet cells.[7]

Islet cell cultures. Islets were isolated from the pancreata of neonatal Sprague-Dawley rats and prepared for culture as described previously.[8] After an initial 16-h incubation in 60-mm culture dishes to remove adherent fibroblastoid cells, suspensions of islet cells were plated into 16-mm culture wells (24 wells/plate). Twenty-four hours after plating, the monolayers were treated for 48 h with serum-free, cysteine-free medium to reduce contamination by fibroblastoid cells.[9] Thereafter, islet cells were maintained using Medium 199

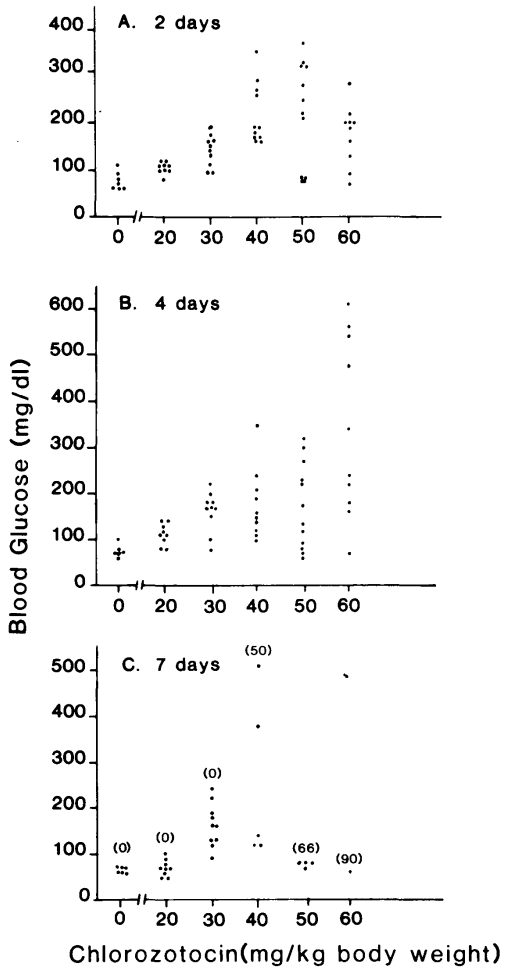

FIGURE 2. Nonfasting blood glucose concentrations in control and CLZ-treated hamsters bled at 2 (A), 4 (B), and 7 (C) days after injection. Numbers in parentheses in C represent the mortality (i.e., percentage of animals in each group) at 7 days.

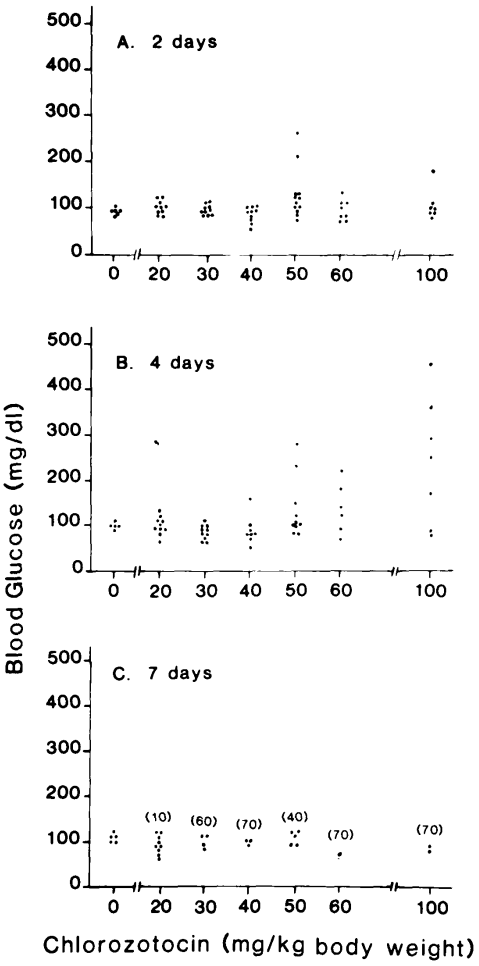

FIGURE 3. Nonfasting blood glucose concentrations in control and CLZ-treated mice. All animals were bled at 2 (A), 4 (B), and 7 (C) days after injection. Numbers in parentheses in C represent the mortality (i.e., percentage of animals in each group) at 7 days.

(Flow Laboratories, McLean, Virginia) supplemented with 10% fetal bovine serum, glucose (300 mM/L), and gentamycin (1 mM/L). Fresh medium was added at 48-h intervals, and the spent medium saved for determination of immunoreactive insulin (IRI) concentration by radioimmunoassay.[8] On the eighth day of culture, STZ and CLZ, alone and with 3-O-MG or 3-aminobenzamide (3-AB) were added to the monolayers. In brief, a 1 mM concentration of STZ or CLZ (Kodak Company, Rochester, New York) was prepared in Hanks' balanced salt solution (HBSS) alone or containing the nonmetabolizable glucose analogue, 3-O-MG (Sigma), or the inhibitor of polyADPribose synthetase, 3-AB (Sigma). The STZ was initially dissolved in citrate buffer (pH 4.5) before dilution in HBSS, whereas CLZ was dissolved in DMSO. The culture medium was removed and the monolayers washed two times with HBSS before exposure to drugs and their proposed inhibitors of action for 1 h. Control cultures received either 0.1% DMSO or citrate, or the drugs or the inhibitors alone. After 1 h, the test chemicals were removed, and the cultures replenished with fresh culture medium. Medium was changed at 48-h intervals thereafter, and the spent medium saved for determination of immunoreactive insulin (IRI).

Radioimmunoassay for insulin. Insulin release during each 48-h period was determined by a back-titration method[10] using ^{125}I-labeled porcine insulin (Cambridge Medical Diagnostics, Inc., Billerica, Massachusetts) diluted with cold porcine insulin (kindly provided by Dr. Ronald Chance, Eli Lilly and Company, Indianapolis, Indiana) and guinea pig anti-bovine insulin serum (Linco Research, Inc., Eureka, Missouri).

RESULTS

Blood glucose. Hamsters became hyperglycemic (defined as 150 mg glucose/dl) 2 days after injection of CLZ at concentrations ranging from 30 to 60 mg/kg (Figure 2). The diabetogenic effect in the hamster was dosage dependent and was most pronounced 4 days after injection. Some animals failed to exhibit elevated concentrations of glucose in the blood even when higher dosages were administered. Death of some hyperglycemic animals was observed between 4 and 7 days (Figure 2C). All of the hamsters exhibiting elevated blood glucose concentrations after the administration of 50 and 60 mg/kg CLZ were dead at 7 days.

Hyperglycemia was observed in only a few mice after injection of CLZ at doses ≥ 50 mg/kg (Figure 3). All diabetic animals died between day 4, the time when hyperglycemia was most prominent, and day 7. In contrast, blood glucose concentrations in rats were normal at 2, 4, and 7 days after administration of CLZ (20–100 mg/kg, data not shown). The relative insensitivity of the rat to the drug was confirmed by glucose tolerance tests on day 4. Abnormal carbohydrate tolerance was observed only after injection of 100 mg/kg CLZ, the approximate LD_{50} dosage in this species (Figure 4).

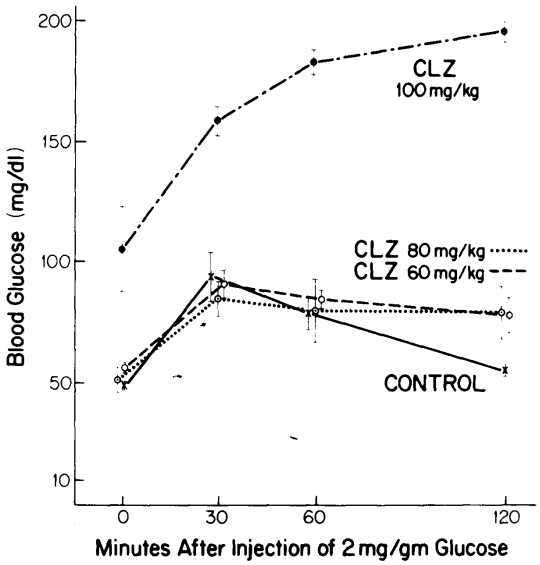

FIGURE 4. Carbohydrate tolerance in control and CLZ-treated rats. Four days after administration of CLZ, fasting rats were injected i.p. with 2 mg/g glucose. Blood glucose concentrations were determined at time 0 and 30, 60, and 120 min after injection of glucose.

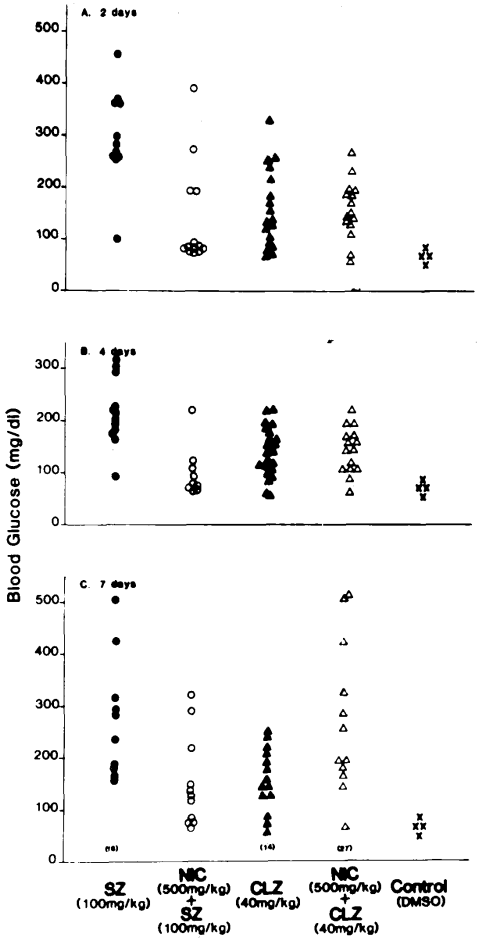

FIGURE 5. Blood glucose concentrations at 2 (A), 4 (B), and 7 (C) days in control, STZ-, and CLZ-treated hamsters after pre-administration of nicotinamide. Numbers in parentheses in C represent the mortality (i.e., percentage of animals in each group) at 7 days. No deaths occurred in control hamsters or in those administered nicotinamide and STZ.

To determine whether preadministration of pyridine nucleotides[5] prevents CLZ-induced diabetes, hamsters were administered nicotinamide (500 mg/kg) i.p. 10 min before injection of either CLZ (40 mg/kg) or STZ (100 mg/kg), the positive control for these experiments. Nicotinamide ameliorated SZ-, but not CLZ-induced hyperglycemia at 2, 4, and 7 days (Figure 5).

Histopathology. Table 1 summarizes observations on the pancreata of animals receiving various dosages of CLZ. The extent of insular necrosis and degranulation of beta cells is expressed on an ordinal scale in which 0 indicates no alteration and 4+ denotes most severe changes. Rats failed to develop lesions of the islets, whereas insular changes were consistently observed in mice and hamsters injected with ≥50 mg/kg CLZ (Figure 6). The beta cells of these animals were often pyknotic, and the granules appeared to be clumped in the cytoplasm. Occasional cells were obviously necrotic and exhibited varying degrees of degranulation. Inflammation of the pancreata was not observed, although the acinar tissue was mildly congested and edematous. Morphologic changes were not found in alpha cells, and there was no evidence of degranulation.

STZ (100 mg/kg) induced severe necrosis and degranulation of beta cells but had no apparent effect on alpha cells of the hamster (Figure 7, A–C). Beta cell degranulation was prominent in diabetic hamsters treated with STZ, whereas degranulation was less striking in CLZ-injected animals. Nicotinamide prevented the necrosis of beta cells caused by STZ, although some degranulation still was evident (Figure 7, D–F). In contrast, nicotinamide did not ameliorate CLZ-induced injury (Figure 7, G–I).

Subtle cytologic changes were evident in the renal tubules of hamsters receiving 30 mg/kg of CLZ and frank epithelial necrosis was observed after injection of 50 mg/kg. Many of these animals had elevated concentrations of blood urea nitrogen, but creatinine was not increased. Focal myocardial necrosis and exudative interstitial pulmonary inflammation were seen in animals receiving the higher dosages. Heart, lung, and kidney of mice and rats appeared to be more resistant to the cytotoxic effects of CLZ, but necrotizing renal tubular changes were observed in rats receiving 100 mg/kg CLZ. Although the cause of death in these rodents is uncertain, the lesions of renal tubules were doubtlessly significant and are consistent with the findings of previous toxicity studies.[11] In this regard, the nephrotoxicity of STZ[12] and other nitrosoureas[13] is well documented in humans. These drugs are eliminated by renal excretion.

Effects of 3-O-MG and 3-AB on STZ- and CLZ-induced damage to cultures of beta cells. To determine whether the mechanism of cytotoxicity of CLZ is similar to that of the known beta cell toxin, STZ, monolayer cultures prepared from the islet cells of neonatal rats were exposed to the two drugs in combination with agents (3-O-MG and 3-AB) that are known to inhibit the effects of STZ on beta cells. The toxicity of CLZ could be blocked in part by the nonmetabolizable sugar 3-O-MG; however, greater concentrations (100 and 150 mM) were required than were necessary to ameliorate the effects of STZ (Figure 8, A and B).

The inhibitor of polyADPribose synthetase, 3-AB, prevented STZ-induced beta cell damage (Figure 9A) but was ineffective in reducing CLZ-induced toxicity (Figure 9B).

TABLE 1
Insular changes in animals receiving various dosages of CLZ*

	CLZ (mg/kg)	Days after inoculation	Insular necrosis	Beta cell degranulation
Mice	100	3	2–4+	±
	50	3–4	1+	±
	25	7	0	0
Hamsters	50	4	3+	2+
	40	7	2–3+	2–3+
	30	4	0–3+	0–2+
Rats	50	7	0	0
	30	7	0	0

*Insular necrosis and degranulation of beta cells was graded using a 4-point ordinal scale in comparison with tissues from rodents receiving 0.1 ml DMSO. Most severe alterations are scored 4+, whereas 0 indicates no change. A ± indicates equivalent positive and negative changes.

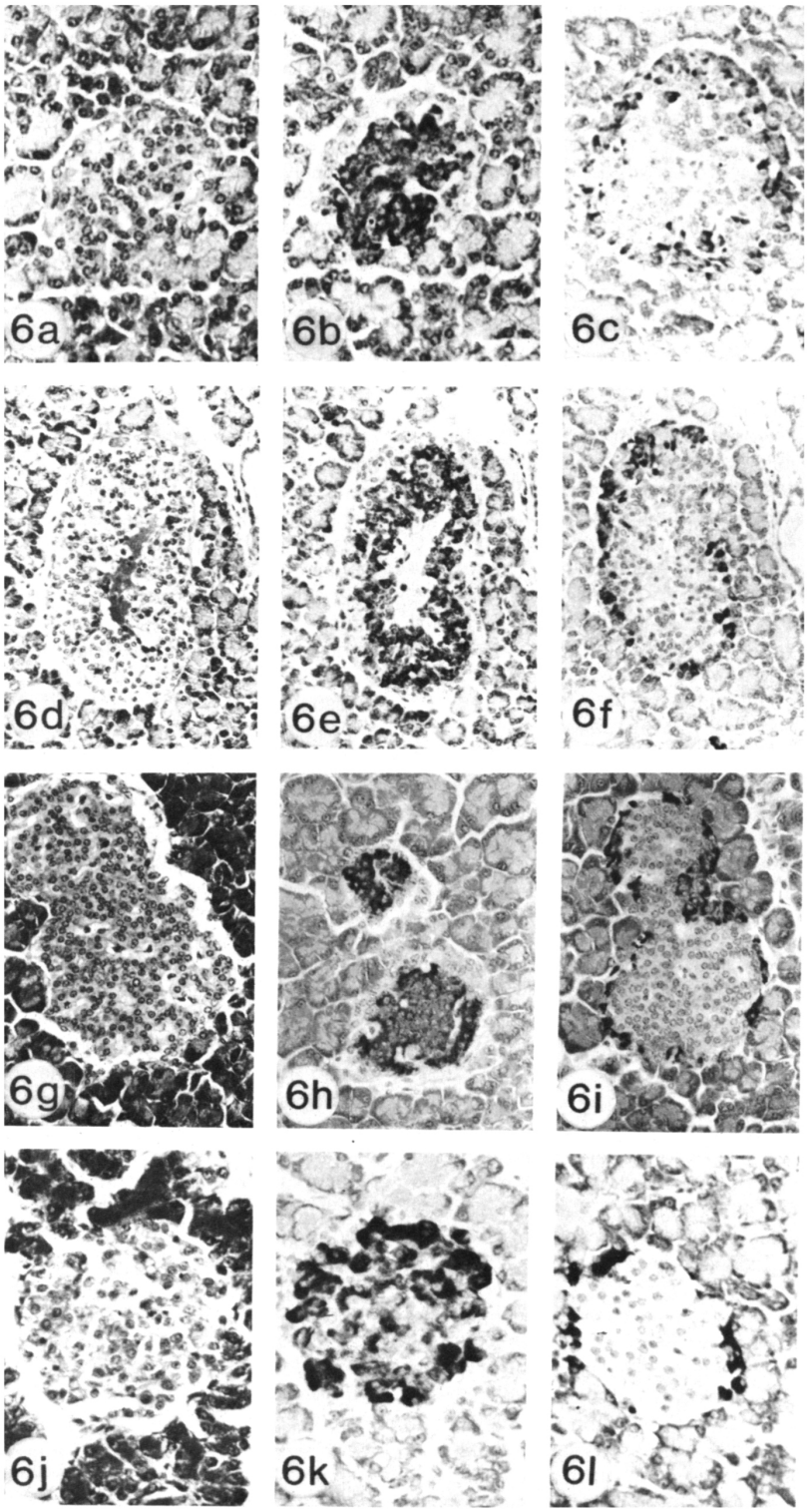

FIGURE 6. Insular alterations in hamsters injected with CLZ (50 mg/kg, A–C, ×148) at 4 days postinjection. After administration of 30 mg/kg CLZ (D–F, ×84), beta cells were affected less severely. Control hamsters receiving DMSO (G–I, ×42) show no insular changes. In rats receiving 100 mg/kg CLZ, islets appear distorted and filled with vacuolated cells (J–L, ×140). Hematoxylin and eosin (A, D, G, and J). The peroxidase-anti-peroxidase technique for insulin (B, E, H, and K), and glucagon (C, F, I, and L) illustrates the affinity of CLZ for the beta cell.

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