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In Vitro Toxicity of Alloxan for Guinea Pig B Cells: Comparison with Rat B Cells (41694)

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Abstract. Previous studies have shown that guinea pigs are resistant to the *in vivo* diabetogenic action of alloxan and that this resistance may be accompanied by a regeneration of B cells in the initial days following administration of the drug. In the studies reported here, we used the measurement of insulin and glucagon released over a 7-day culture period as indices of islet cell viability and examined effects of *in vitro* exposure to alloxan upon subsequent release of insulin and glucagon from guinea pig (alloxan-resistant) and rat (alloxan-sensitive) islet cell cultures. An alloxan dose-dependent decrease in subsequent insulin release was found. However, whereas the lowest concentration of the drug (1 m*M*) produced a significant depression in insulin release in rat islet cultures, with maximal depression occurring after exposure to 5 m*M* alloxan, insulin release from guinea pig cultures was not significantly depressed by 1 or 2 m*M* alloxan, and 5 m*M* alloxan treatment produced a submaximal depression. Furthermore, insulin release from guinea pig but not rat cultures increased transiently at between 6 and 18 hr during the first day following exposure to all doses of alloxan. Treatment with high doses of the drug (40 m*M* or greater) caused the same maximal chronic depression of insulin release for both species. In contrast, glucagon release from cultures of both species was not affected significantly following alloxan treatment. Thus, guinea pig B cells are more resistant than those of the rat to the action of alloxan, but this resistance can be overcome by employing high doses of the drug. Other factions unidentified by the present studies may also be involved in the failure of guinea pigs to develop diabetes following *in vivo* treatment with alloxan.

The guinea pig is a relatively unique species in its resistance to the diabetogenic action of the B cell cytotoxic compound alloxan (1). Previous studies of guinea pigs treated *in vivo* with high doses of alloxan (2–5) have suggested that the B cells of this species may be initially destroyed by the drug but rapidly regenerate thereafter, thus precluding the development of permanent diabetes. However, it is unknown to what extent a resistance of the B cells themselves to the cytotoxic action of alloxan may contribute to the failure of these animals to become diabetic. To examine this possibility, we chose to measure insulin and glucagon release by islet cell cultures over a 7-day period as indices of islet cell viability, and compared the effects of a brief 5-min exposure to alloxan upon insulin and glucagon release by guinea pig (alloxan-resistant) and rat (alloxan-sensitive) islet cell cultures.

Materials and Methods. Male albino guinea pigs weighing 300–400 g were purchased from Charles River Laboratories and female albino rats weighing 200–250 g were purchased from Tyler Labs. For each experiment, islets were isolated from the pancreata of each of four animals using a collagenase digestion technique (5) (type IV collagenase from Worthington Biochemicals), modified from the procedure described by Lacy and Kostianovsky (6), with over 100 islets being obtained from each animal. The islets were aseptically collected under a microscope into phosphate-buffered saline (PBS) containing 50 μg/ml gentamycin, 100 μg/ml neomycin, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 U/ml polymyxin B, and 3 μg/ml amphotericin B. All antibiotics were from GIBCO except gentamycin (Microbiological Associates). Following sedimentation, the islets were rewashed in this antibiotic solution and then dispersed into single cells and small cell clusters by incubation for approximately 3 min at 37°C in 10 ml of a solution of 0.25% trypsin (1:250, Difco) and 2 m*M* ethylenediaminetetraacetate (EDTA) (Mallinckrodt) in Ca- and Mg-free PBS. The reaction was stopped by diluting the trypsin-containing solution with an equal volume of tissue culture medium RPMI 1640 (GIBCO) containing 10% (v/v) heat-inacti-

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vated fetal bovine serum (GIBCO), 50 μg/ml gentamycin, and 300 mg/dl glucose (=culture medium). The cells were collected by centrifugation for 5 min at 2000 rpm and were suspended in 20–25 ml of culture medium. One milliliter of the suspension was added to each of 20 35 × 10-mm petri dishes (Lux Scientific) along with 0.5 ml of additional culture medium. The dishes were numbered randomly and incubated undisturbed for 3 days in a humidified atmosphere of 5% CO_2–95% air at 37°C. This allowed cell attachment to occur. The medium was replaced with 1.5 ml of fresh culture medium and the dishes were incubated for 24 hr prior to collection of medium for measurement of pretreatment rates of immunoreactive insulin and glucagon release. In order to inhibit proteolytic degradation of glucagon, 50 μl/ml of 1.0 *M* benzamidine was added to samples for glucagon measurement (7). Samples for both insulin and glucagon measurements were stored at −20°C prior to assay. Rat insulin, rat and guinea pig glucagon (8), and guinea pig insulin (9) were measured by previously described methods.

Alloxan (Nutritional Biochemicals Co.) was prepared as a 600 m*M* stock solution in McIlvaine buffer, pH 2.9. Following aseptic removal of culture medium for subsequent measurements of basal rates of hormone release, and replacement with 1.9 ml of minimal essential medium (MEM) (Gibco) containing 0.5% (v/v) heat-inactivated fetal bovine serum, cultures of rat and guinea pig islet cells were treated with alloxan in triplicate. Reciprocal volumes of buffer and alloxan stock solutions totalling 100 μl were added to each culture to produce final alloxan concentrations in the dishes of 0, 1, 2, 5, 40, or 60 m*M*. The dish contents were swirled immediately after addition of alloxan solution, followed by incubation for 5 min at room temperature before the alloxan-containing medium was quickly removed and replaced with 1.5 ml of fresh culture medium. The dishes were then incubated for 24 hr following which medium was collected for measurement of hormone content ("Day 1" following treatment with alloxan). Following replacement with fresh culture medium, dishes were incubated sequentially for additional 48-hr intervals with collection of medium samples for hormone assay and replacement with fresh culture medium on "Day 3," "Day 5," and "Day 7" after alloxan treatment.

In another study, quadruplicate culture dishes of guinea pig islet cells were exposed to 0, 5, or 40 m*M* alloxan and medium samples collected at 2, 6, 12, 18, 24, and 72 hr following exposure to alloxan for measurement of insulin content.

To determine whether nonspecific desquamation from the cell layer might contribute to the lowering of insulin values following alloxan exposure, the DNA contents of quadruplicate cultures of guinea pig islet cells were determined at 5 min and 24 hr following exposure to either 0, 5, or 40 m*M* alloxan (10). DNA content of a pretreatment group was also measured.

Dispersed guinea pig islet cells were also added to culture slide-chambers (Lab-Tek Products, tissue culture chamber/slides, 4 chambers/slide), with each chamber receiving about 25% of the number of cells added to each 35 mm tissue culture dish. Quadruplicate slide/chambers were preincubated for 2 hr with MEM containing 30 mg/dl glucose, then fixed and immunostained for insulin on Days 1 and 3 following a 5-min exposure to either 0, 5, or 40 m*M* alloxan. For immunocytochemistry, a peroxidase–antiperoxidase method was used (11) with guinea pig anti-porcine insulin serum diluted (1:50) as the primary antiserum. Appropriate immunocytochemical controls were used, including omission of primary antiserum, omission of all antisera, substitution of diluted (1:100) normal serum for primary antiserum, or using primary antiserum previously mixed with porcine insulin 100 μg/ml. Numbers of immunostained B cells were counted in 40 microscopic fields (200× magnification) per chamber.

Analysis of data was performed by means of the split plot in time model of the analysis of variance (12) run on a CDC 6000 computer utilizing the SPSS-MANOVA program. Comparisons among means were performed using orthogonal (13) or nonorthogonal (14) contrasts, since this procedure does not result in erroneously inflated numbers of significant differences for multiple comparisons among means as occurs with Student's *t* test and related procedures (15).

Results. An alloxan dose-dependent decrease in insulin release was observed for both guinea pig (Fig. 1) and rat (Fig. 2) islet cell cultures. However, on the first day following exposure of guinea pig islet cell cultures to all doses of alloxan, insulin release was transiently increased. This was not observed with rat cultures. Furthermore, rat cultures were much more sensitive to alloxan than guinea pig cultures. One millimolar alloxan caused significant depression of insulin release in rat islet cultures, 2 m*M* alloxan was almost maximally effective, while 5 m*M* alloxan exerted a maximal effect. In guinea pig islet cultures, 1 m*M* alloxan was ineffective, 2 m*M* alloxan was only slightly and inconsistently effective, and 5 m*M* alloxan caused a submaximal effect. However, the two highest concentrations of alloxan, 40 and 60 m*M*, produced the same maximal depression in insulin release in both types of cultures. In addition, rates of insulin release remained relatively constant for the entire period of study once they had been depressed by effective concentrations of the drug.

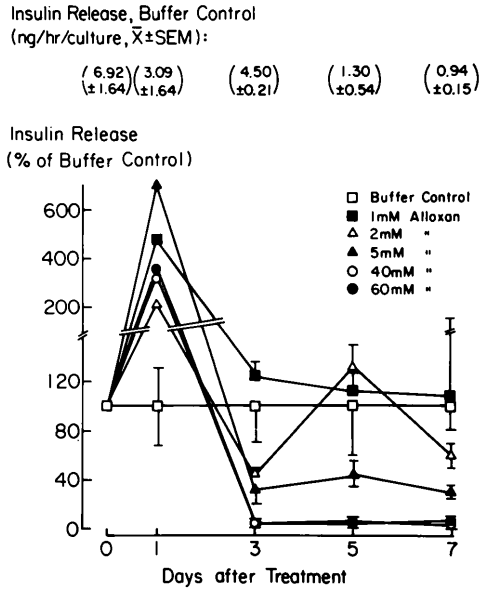

FIG. 1. Insulin release rates for triplicate cultures of guinea pig islet cells treated for 5 min with one of five different doses of alloxan on Day 0. In this and subsequent figures, the absolute release rates for control (buffer-treated) cultures are given at the top in parentheses, while relative release rates (percentage of buffer control, means, and SEM) are shown in the graph. SEM are not shown for data on Day 1 due to complete overlap of SEM for the alloxan-treated groups.

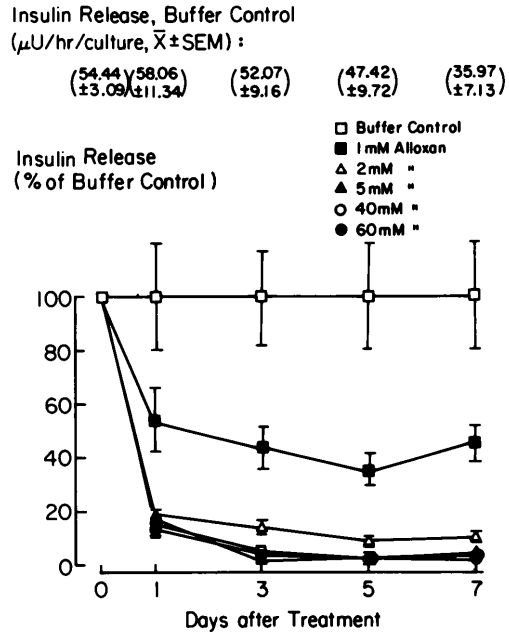

FIG. 2. Insulin release rates for triplicate cultures of rat islet cells treated for 5 min with one of five different doses of alloxan on Day 0. Please refer to legend, Fig. 1, for further explanation.

In contrast to the potent effects of alloxan treatment upon culture insulin release, there was little effect of alloxan upon subsequent glucagon release from either guinea pig (Fig. 3) or rat (Fig. 4) islet cell cultures. Although glucagon release from rat cultures increased in the initial days after alloxan exposure, whereas release from guinea pig cultures was unaltered, the differences between the species was not significant when pooled across all time points.

In guinea pig islet cell cultures, the increase in insulin release after exposure to alloxan occurs as early as 6 hr (Fig. 5). The increase was greater with 40 than with 5 m*M* alloxan. In this experiment, although a transient increase in insulin release was observed, insulin release was normal at 3 days after 5 m*M* alloxan, in contrast to the experiment shown in Fig. 1 where insulin release was significantly reduced at 3 days after 5 m*M* alloxan. This difference is probably related to 5 m*M* alloxan being a dose with intermediate effectiveness and the lability of alloxan, even in the acid

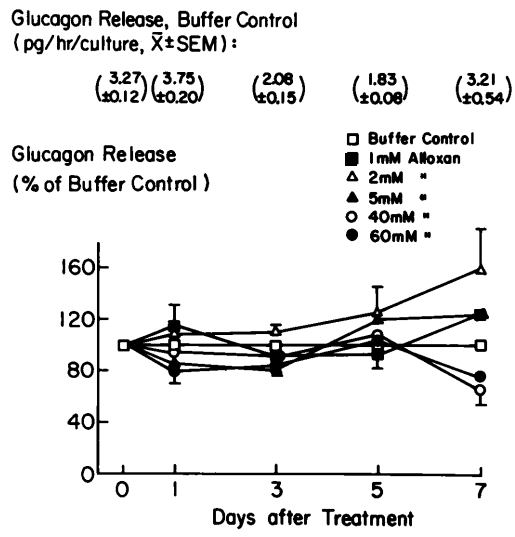

FIG. 3. Glucagon release rates for triplicate cultures of guinea pig islet cells treated for 5 min with one of five different doses of alloxan on Day 0. Please refer to legend, Fig. 1, for further explanation. Only selected SEM are provided due to complete overlap among groups.

conditions used to treat the cultures. In the cultures exposed to 40 m*M* alloxan, insulin release fell to less than 20% of control levels by 3 days.

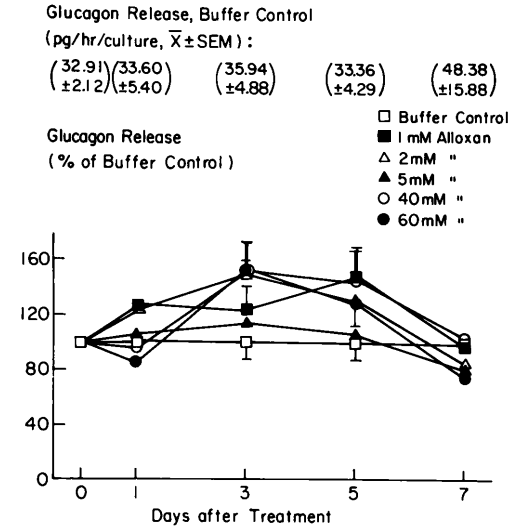

FIG. 4. Glucagon release rates for triplicate cultures of rat islet cells treated for 5 min with one of five different doses of alloxan on Day 0. Please refer to legend, Fig. 1, for further explanation. Only selected SEM are provided where groups were not overlapping.

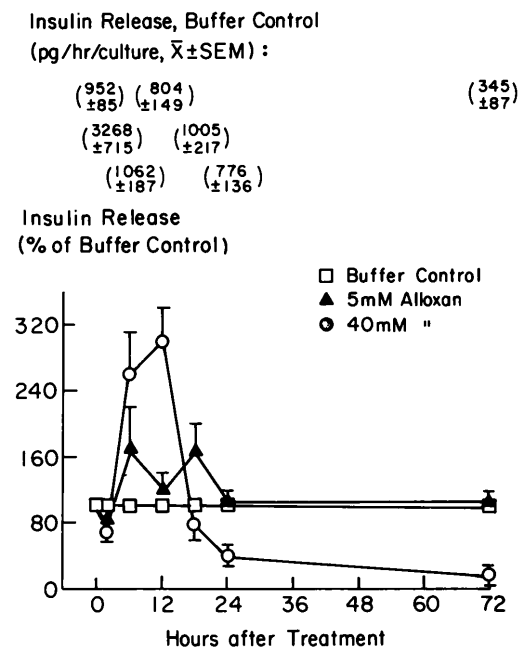

FIG. 5. Insulin release rates for cultures of guinea pig islet cells treated for 5 min with one of two different doses of alloxan ($N = 8$ for each group) at time 0. Please refer to legend, Fig. 1, for further explanation.

Treatment of guinea pig islet cell cultures with alloxan did not cause any significant effects upon DNA content (Fig. 6). The doubling of DNA content which occurred at 24 hr ($P < 0.001$) was almost entirely attributable to an increase in nonendocrine cells in these cultures.

The number of B cells fell significantly after exposure of guinea pig islet cell cultures to alloxan (Fig. 7). On Day 1 following exposure to alloxan, cultures treated with 5 m*M* alloxan were not significantly reduced in numbers of B cells as compared to buffer treatment but were significantly affected on Day 3 ($P < 0.01$). Cultures treated with 40 m*M* alloxan had significantly reduced numbers of B cells on both Days 1 and 3, when compared to either buffer treated cultures or cultures treated with 5 m*M* alloxan ($P < 0.01$).

Discussion. The present studies demonstrate the utility of employing alloxan treatment of cultured islet cells as a means of assessing the intrinsic sensitivity of islet cells of different species to the toxic action of the drug. Guinea pig islet B cells appear to be more

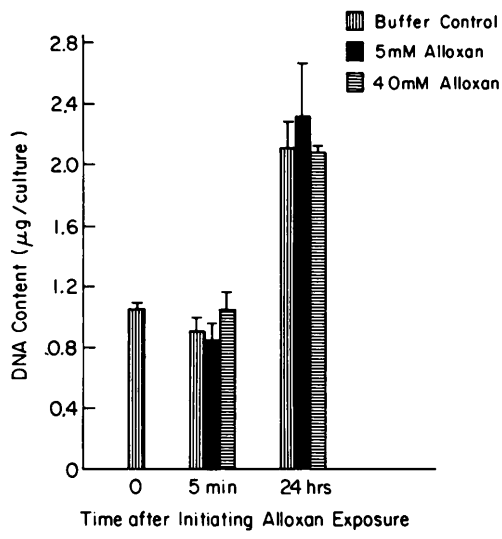

FIG. 6. DNA contents (mean and SEM) of quadruplicate cultures of guinea pig islet cells treated for 5 min with one of two different doses of alloxan at 0 time, measured at 5 min and 24 hr after exposure of culture to alloxan. A pretreatment group (0) is also shown.

resistant than rat B cells to the depressive effect of a brief period of exposure of cells to alloxan. The toxicity of alloxan to islet B cells was determined by measurements of rates of insulin release over a subsequent 7-day culture interval. In contrast to the low threshold of rat B cells for this effect (≤ 1 m*M* alloxan), insulin release from guinea pig cultures was not depressed by alloxan concentrations below 5 m*M*. At this latter concentration, the effect

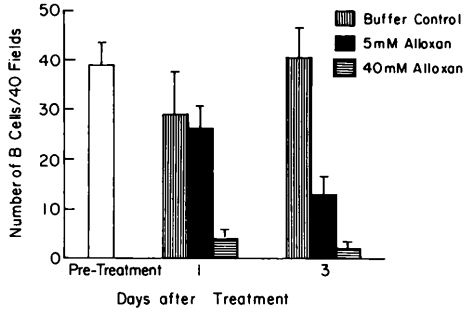

FIG. 7. Number of immunostained guinea pig B cells counted in 40 microscopic fields (200×) per culture slide chamber. Quadruplicate cultures were treated for 5 min with one of two different doses of alloxan and following fixation on Days 1 and 3 after exposure to alloxan, were stained for insulin by the peroxidase–antiperoxidase technique.

upon insulin release by guinea pig cultures was submaximal, whereas even 2 m*M* alloxan produced a nearly maximal depression of insulin release by rat cultures. However, this resistance of guinea pig B cells to alloxan was dose-dependent and could be overcome by greater concentrations of the drug. The implication of hydroxyl radicals (16–18), peroxides (19, 20) and/or membrane thiol groups (21–23) in the mechanism of alloxan cytotoxicity suggest possible further areas of research in order to ascertain the mechanisms for differences in sensitivity between guinea pig and rat B cells to alloxan effects.

The absence of an increase in insulin response from the alloxan-treated cells during the subsequent 7-day culture period is consistent with a permanent effect of the drug on the B cells, as suggested by a decrease in B cells per culture, rather than an effect upon insulin release per se resulting from known and presumably reversible effects of alloxan, such as inhibition of insulin biosynthesis and release (24, 25).

The fact that the major depression in insulin release from rat islet cell cultures occurred within the first 24 hr following exposure to alloxan is similar to our findings in alloxan-treated neonatal rat pancreatic monolayer cultures (26) and is consistent with the occurrence of B cell necrosis and dissolution within the first day following alloxan treatment *in vivo* (27). In contrast, the major decline in insulin release from guinea pig islet cell cultures following treatment with effective concentrations of alloxan did not occur until after the first 24 hr following exposure to the drug, and a significant increase in insulin release above control levels occurred between 2 and 18 hr. At least four explanations can be offered for the differential responsiveness of these two species early after alloxan treatment: (1) Cultured guinea pig B cells may store more insulin than rat B cells, and therefore more insulin would be released when the cells are destroyed during the first day after alloxan treatment. (2) The methods used to assay guinea pig vs rat insulin may be differentially sensitive to cross-reaction by proinsulin, insulin degradation products, or other molecules which may be liberated upon destruction of the B cells by alloxan. (3) Guinea pig B cells may continue to synthesize and release insulin at

near normal rates for a greater period after alloxan exposure than those of the rat. This prolongation of survival would lead to a greater average 24-hr rate of insulin release than if the cells were killed soon after the drug treatment. (4) The early increase in insulin release from guinea pig B cells may represent a longer period of transient stimulation of insulin release by alloxan whereas this stimulatory effect of the drug appears to be very brief for rat B cells (28, 29).

In contrast to the acute inhibition of rat islet A cell function reportedly produced by alloxan (28), glucagon release during the 7 days following alloxan treatment was not depressed below control levels for either rat or guinea pig islet cell cultures. These results are similar to our findings in neonatal rat pancreatic monolayer cultures treated with alloxan (26), and indicate that this drug is not permanently cytotoxic for A cells even at the highest doses (60 m*M*) employed in these studies. Our results differ somewhat from those of Ostenson (30) who reported an acute inhibitory effect of alloxan on A-cell-enriched guinea pig islets in culture, but abnormalities of glucagon release disappeared following 1 week in culture. Thus the depressive and presumably cytotoxic action of alloxan upon B cell hormone release appears to be specific for this cell type.

As alloxan had no significant effect upon DNA content of islet cell cultures, nonspecific cellular desquamation following alloxan exposure is unlikely to be the major determinant of these observations. Since B cells make up a relatively small percentage of the total cell population of these cultures, it is necessary to specifically identify and count B cells in order to determine changes in the B cell population. By doing this, on the first day following exposure to 5 m*M* alloxan the number of immunostained B cells was similar to control values, while B cells were significantly reduced after treatment with 40 m*M* alloxan. On the third day after exposure to alloxan, B cells were reduced for both the 5 m*M* and 40 m*M* alloxan-treated cultures. These changes roughly paralleled changes in insulin secretion (compare Figs. 1, 5, and 7). These data strongly suggest that the changes in insulin release rates are indeed reflective of changes in B cell numbers following alloxan exposure.

In summary, these studies indicate that insulin release by islet cell cultures over a 7-day period following exposure of the cells to alloxan may be used as an index of B-cell sensitivity to the toxic effects of the drug which are longer lasting. B cells of the guinea pig islet were relatively more resistant to alloxan than rat B cells, suggesting a possible reason for the resistance of guinea pigs to the diabetogenic action of alloxan. The exact mechanism responsible for this species difference remains to be elucidated. In contrast to B cells, islet A cells are relatively insensitive to alloxan in both species.

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