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Pancreatic B cells possess defense mechanisms against cell-specific toxicity

(diabetes/alloxan/streptozotocin/islet cell surface antibodies)

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ABSTRACT **Insulin-dependent diabetes develops when more than 90% of the insulin containing B cells are destroyed. The present study investigates whether the target B cells can counteract the damaging effects of cytotoxic substances. Purified islet cells were first exposed for 3–10 min to *t*-butylhydroperoxide, alloxan, streptozotocin, or B-cell surface antibodies plus complement, then cultured for 20 hr before the percent of dead cells was counted. *t*-Butylhydroperoxide destroyed all islet cell types whereas the three other agents exerted a dose-dependent toxicity upon islet B cells only. The survival of drug- and complement-treated cells varied with the culture conditions present between the initial cellular attack and the moment of cell death. For the four B-cell toxic agents tested, an increase in medium glucose following any of these treatments reduced the percent of dead cells. This protective effect was not observed with galactose or fructose, nor could it be induced in islet non-B cells; it was additive to the protective action glucose induced during preincubation of the cells prior to their exposure to certain cytotoxic agents such as alloxan. Nicotinamide also enhanced the survival of drug-treated B cells, irrespective of the damaging compound. The vitamin was most effective when applied immediately after the initial drug or complement treatment; it also protected islet non-B cells—in contrast to glucose. The present *in vitro* study has led to the recognition of defense mechanisms in pancreatic B cells. Physiologic compounds such as glucose and nicotinamide were found to stimulate islet B cells to counteract the damaging effects of B-cell toxic conditions. It is conceivable that the events involved in this protection are implicated in the pathogenesis and/or prevention of insulin-dependent diabetes.**

Insulin-dependent diabetes is caused by a marked reduction in the number of pancreatic B cells (1), probably as a result of a viral, chemical, and/or autoimmune attack on the cells (2–8). The destructive process may be fulminant and of short duration (3, 5) or—as in most cases—extended over months or years (9, 10). If more than 90% of the insulin-containing B cells are affected, diabetes is expected to develop (1), while a less marked cell loss can be responsible for a state of glucose intolerance (10) or may remain undetectable. This variability in length and extent of B-cell destruction can be related to the severity of the cytotoxic conditions but may also reflect differences in the susceptibility of the target cells. The diabetogenic effect of experimental B-cell killers such as alloxan and streptozotocin is, for example, reduced by pretreating the target cells with substances that are thought to neutralize the initial effects of the drugs (11–15). Furthermore, the knowledge that the moment of cell death often occurs several hours or days after this initial attack (16) raises the question whether the degree of cell destruction can also vary with the activity of hypothetical defense mechanisms that are inducible in treated cells. To investigate the participation of cellular defensive reactions, we have employed purified pancreatic B cells in an *in vitro* model system wherein cytotoxicity is measured as the percent of dead cells 20 hr after their short exposure to a (potential) diabetogenic agent. The existence of inducible defense mechanisms is then tested by comparing cell survival after culturing the diabetogenic agent-treated cells in media of different composition. The specificity of such protective events is determined by assessing their activation following treatment with four different cytotoxic agents and by examining their occurrence in other islet cell types.

MATERIALS AND METHODS

Preparation of Purified B and Non-B Islet Cells. Pancreatic islets were prepared from adult Wistar rats (200–250 g body weight) by collagenase digestion of the glands (17, 18). Islet-cell suspensions were obtained by a mild mechanical treatment of the isolated islets in calcium-free medium containing trypsin and DNase (18, 19). After a 20-min incubation in CMRL-1066 (GIBCO) supplemented with 0.5% bovine serum albumin (Sigma), the cells were centrifuged through a layer of Percoll (density, 1.04 g/ml) to remove debris and dead cells (19). After resuspension in Hepes-buffered Earle's medium containing 0.5% bovine serum albumin (EH, pH 7.35; for composition see ref. 19), the islet cells were purified by autofluorescence-activated cell sorting in a FACS-IV (Becton Dickinson) (18, 20). This technique has been shown to separate single insulin-containing B cells from single islet non-B cells on the basis of their higher light scattering activity and their FAD content at 2.8 mM glucose (18, 20). The method is usually applied to 20 rat pancreata and yields 10^5 B cells and 7×10^4 non-B cells per pancreas with a cross contamination of less than 5% (18).

Exposure to Drugs. Immediately after their separation, purified islet B and non-B cells were distributed over 400-μl samples of 10^4 cells and preincubated for 15 min at 37°C in EH with or without glucose. The cells were then exposed to alloxan (Sigma) or streptozotocin (Upjohn) for 3 min or to *t*-butylhydroperoxide for 5 min. The drugs were administered at various concentrations in either 5 μl of 1 mM HCl (for alloxan and streptozotocin) or 5 μl of ethanol (for *t*-butylhydroperoxide). Control cells were treated with solvent only. At the end of the indicated periods, the cells were washed twice with EH without glucose, resuspended in CMRL-1066, and cultured in polylysine-coated microtiter cups (1500 cells per cup) (21).

Islet B cells, treated with alloxan or streptozotocin, were also analyzed in a fluorescence-activated cell sorter (FACS IV) equipped with two argon lasers (164-06 and 171-17, Spectra Physics, Mountain View, CA). Under these conditions, the effect of glucose upon pancreatic B cells can be

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rapidly monitored via variations in the cellular FAD- and NAD(P)H-autofluorescence intensities (20, 22).

Exposure to Islet Cell Surface Antibodies and Complement. Sera from recently diagnosed insulin-dependent diabetics were examined for the presence of islet cell surface antibodies as described (23, 24). Analysis was carried out after ammonium sulfate precipitation and dialysis against phosphate-buffered saline (24). A sample with a high titer of B-cell surface antibodies was selected for cytotoxicity testing; immunoglobulins from a nondiabetic patient without detectable islet cell surface antibodies served as control. Islet cells were first incubated for 60 min at 37°C in a total volume of 200 μl of EH containing serum immunoglobulins at a final dilution of 1:100. The incubation was continued for 10 min at 37°C after addition of 20 μl of complement (final dilution 1:11, Low-tox-M rabbit complement, Cedarlane Laboratories, Hornby, ON, Canada). The cells were then washed twice in EH, resuspended in CMRL-1066, and transferred into polylysine microtiter cups (1500 cells per cup) for a 20-hr culture period.

Culture Conditions. Polylysine-fixed cells were cultured for 20 hr at 37°C in a humid atmosphere of 7.5% CO_2/92.5% air (Forma CO_2 incubator). Basal culture medium consisted of 200 μl of CMRL-1066 containing 5.6 mM glucose and 10% (vol/vol) heat-inactivated fetal calf serum. In certain test conditions, the culture medium was supplemented with 0.2 mM nicotinamide or 14.4 mM of glucose, galactose, or fructose.

Measurement of Cell Toxicity. The percent of dead cells was determined by counting the percent of cells that were not stained by the vital stain neutral red (0.01% final concentration).

Immediately after their isolation, more than 95% of the purified islet cells stained positively with neutral red. In the concentration range of the present experiments, the cytotoxic drugs used did not immediately affect the cellular capacity to accumulate the vital dye, but over the next 15 hr the drugs elicited a progressive decline in this parameter. For example, a 5-min exposure to 5 mM *t*-butylhydroperoxide reduced the number of neutral red positive cells by less than 10% after 1 hr, by 20% after 2 hr, and by more than 50% after 15 hr; no further decrease occurred at later time points. In control samples more than 85% of the cells were deeply stained by neutral red after 20 hr of culture. All cell toxicity measurements were, therefore, carried out 20 hr after exposure to cytotoxic conditions. By this time, regardless of the experimental condition, more than 90% of the initially plated cells were recovered at the bottom of the polylysine-coated cups (total cell numbers were determined by a semi-automatic quantification method, see ref. 25). The percent of neutral red negative cells counted at the end of the 20-hr culture period thus represents a valid index for the cytotoxicity of a particular agent. As the percent of unstained cells in the untreated control samples varied between 3 and 15%, the toxicity of a tested culture condition, X, was calculated as

$$\frac{\%\text{ dead cells in X} - \%\text{ dead cells in C}}{100 - \%\text{ dead cells in C}} \times 100,$$

where C is the corresponding control culture condition.

The listed or plotted toxicity data represent means of these calculated values ± SEM from five to eight experiments. By definition, the toxicity of X is zero in the control cells. The statistical significance of differences was determined by paired Student's *t* testing of the experimental data (i.e., the counted percentages).

RESULTS

Four Different Conditions Leading to B-Cell Death *in Vitro*. The B-cell toxicity of the four selected sets of experimental conditions was determined on purified B cells incubated in glucose-free media before and during exposure to the various agents, and subsequently cultured for 20 hr in medium with 5.6 mM glucose, before the percent of dead cells was counted.

A 3-min exposure to alloxan (0.2–2 mM) or to streptozotocin (2 and 5 mM) elicited a dose-dependent death of islet B cells (Fig. 1). This was also the case for cells incubated for 5 min with *t*-butylhydroperoxide (2 and 5 mM) (Fig. 1).

Addition of complement to cells that had been preincubated with B-cell surface antibodies (final dilution 1:100) led to 62.0 ± 8.6% neutral red negative cells ($n = 5$) versus 8.2 ± 1.6% in control preparations treated with complement and islet cell surface antibody-negative immunoglobulins ($n = 5$).

Cell Specificity of Cytotoxic Conditions. The cell specificity of the four sets of B-cell toxic conditions was assessed by measuring their effect upon the survival of islet non-B cells.

Under the conditions described above, alloxan and streptozotocin were not cytotoxic to islet non-B cells, whereas *t*-butylhydroperoxide elicited higher cell death among non-B cells than B cells (Fig. 1). Treatment of islet non-B cells with the selected islet cell surface antibody-positive serum sample and complement did not induce higher cell damage (9.3 ± 1.7% dead cells) than in control preparations treated with islet cell surface antibody-negative immunoglobulins (8.9 ± 1.5% dead cells; $n = 5$).

These results indicate that among the four conditions of islet B-cell toxicity, three appear to be B-cell specific while one—*t*-butylhydroperoxide—is toxic for all islet cell types.

Effect of Culture Conditions on Survival of Drug- and Complement-Treated Islet Cells. The effect of culture conditions upon the survival of drug- and complement-treated islet cells was first tested after *t*-butylhydroperoxide treatment. When the drug-treated cells were cultured at 20 mM glucose, fewer B cells were neutral red negative by a factor of 2 than at 5.6 mM glucose (Fig. 1). This was not the case when the medium was supplemented with 14.4 mM galactose or fructose instead of 14.4 mM glucose (data not shown). An elevated glucose concentration during culture did not reduce the cytotoxic effects of *t*-butylhydroperoxide on islet non-B cells (Fig. 1). The addition of 0.2 mM nicotinamide to the basal culture medium not only exerted a protective effect similar to that of 20 mM glucose in *t*-butylhydroperoxide-treated B cells but also induced—in contrast to glucose—better survival of *t*-butylhydroperoxide-treated non-B cells (Fig. 1).

Glucose, as well as nicotinamide, promoted the survival of islet B cells that had been exposed to the B-cell specific poisons alloxan or streptozotocin (Fig. 1). The most marked effect was measured in streptozotocin-treated cells cultured with 0.2 mM nicotinamide—this condition counteracted completely the B-cell killing effect of 2 mM streptozotocin and reduced the number of dead B cells by a factor of 7 following exposure to 5 mM streptozotocin (Fig. 1).

Under the condition of complement-induced antibody-dependent B-cell killing, only the combination of 20 mM glucose plus 0.2 mM nicotinamide was tested. Culturing the antibody plus complement-treated cells under these conditions reduced the number of dead B cells by 22%, provided that both agents were also present during the complement reaction (48.2 ± 6.0% dead cells versus 62.0 ± 8.6% when cultured in 5.6 mM glucose—$P < 0.05$, $n = 5$).

It is thus clear that in each of the four conditions of B-cell toxicity, the percent of dead cells is not only determined by the severity of the toxic condition but also by the intensity of

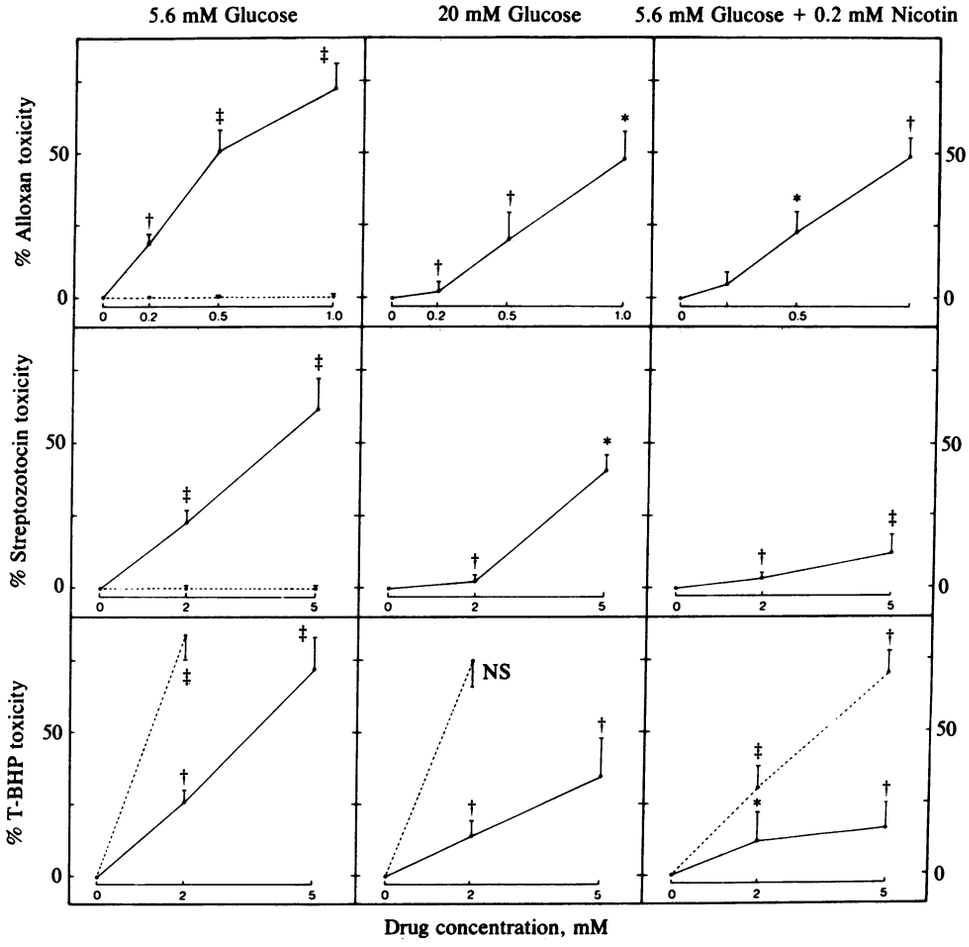

FIG. 1. Toxicity of alloxan, streptozotocin, and *t*-butylhydroperoxide (T-BHP) in islet B (solid lines) and non-B (dashed lines) cells. The cells were preincubated without glucose before exposure to the drugs. After washing, the cells were cultured with 5.6 mM glucose, 20 mM glucose, or 5.6 mM glucose plus 0.2 mM nicotinamide. At the end of a 20-hr culture period, the percent of neutral red negative cells was counted and expressed as net toxicity. Data express the mean net toxicity ± SEM calculated from 3 to 9 separate experiments. The percent of dead cells counted after culture at 5.6 mM glucose was compared to that in the corresponding control preparation (13.5 ± 1.6% dead cells) and the statistical significance of differences calculated by unpaired Student's *t* testing. The percent of dead cells counted after culture in 20 mM glucose or 5.6 mM glucose plus 0.2 mM nicotinamide was compared to that observed in an identical condition cultured at 5.6 mM glucose, and the statistical significance of the differences was calculated by unpaired Student's *t* testing. The percent of dead cells in the control preparations cultured with 5.6 mM glucose differed significantly from that with 20 mM glucose (8.6 ± 0.8%—$P < 0.02$) but not from that at 5.6 mM glucose plus nicotinamide (12.9 ± 3.0%). NS, not significant; $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$.

(the) cellular defense mechanism(s) that can be activated after the initial cellular attack.

Comparison of Glucose Protection Before and After Cytotoxic Attack. The observation that glucose activates the defense mechanism(s) in drug- and complement-treated B cells raised the question whether this cellular protection was related to that obtained by pretreating the B cells with glucose prior to alloxan exposure (26, 27).

The protective effect of culturing drug-treated B cells with 20 mM glucose was, therefore, compared with the effects of preincubation with 20 mM glucose.

When pancreatic B cells were incubated in 20 mM glucose prior to and during exposure to 0.5 mM alloxan, 80% fewer B cells died during subsequent culture in basal medium than in B-cell preparations that were not preincubated with glucose (Table 1). A similar glucose pretreatment did not reduce the cytotoxicity of 5 mM streptozotocin (Table 1). However, the presence of 20 mM glucose in the period immediately following the B-cell exposure to these drugs protected against not only alloxan but especially streptozotocin (Fig. 1). The protective effect of a glucose pretreatment prior to alloxan exposure was additive to that induced by 20 mM glucose during culture of treated cells (data not shown).

The protective glucose pretreatment probably prevents a cellular oxidation immediately after toxic drug exposure. Within 3 min, alloxan (0.5 mM) reduced the cellular NAD(P)H content by more than 80% in cells preincubated without glucose (Table 1). After glucose pretreatment, the mean cellular NAD(P)H content was 2.5-fold higher and only marginally reduced by alloxan (Table 1). In contrast to alloxan, streptozotocin (5 mM during 3 min) had no effect on the NAD(P)H content of B cells preincubated without glucose; in this condition, glucose pretreatment did not decrease the cytotoxicity (Table 1).

DISCUSSION

Animal models of spontaneous or experimental diabetes have permitted the identification of conditions that prevent the

Table 1. Effects of alloxan and streptozotocin upon pancreatic B cells

Test condition	Cellular NAD(P)H, % control fluorescence intensity (VC)	Toxicity, % dead cells	*P*
No glucose			
Control	100 (34)	0	
Alloxan, 0.5 mM	64 (39)	51.0 ± 6.1 (9)	
Streptozotocin, 5 mM	107 (37)	59.5 ± 7.2 (9)	
20 mM glucose			
Control	251 (44)	0	
Alloxan, 0.5 mM	238 (36)	9.0 ± 3.2 (4)	<0.001
Streptozotocin, 5 mM	233 (35)	71.3 ± 8.3 (4)	NS

Isolated B cells were preincubated for 15 min with or without 20 mM glucose and then exposed for 3 min to alloxan or streptozotocin before their NAD(P)H fluorescence intensity was measured in the cell sorter (22). Results are expressed in arbitrary units; the variation coefficient, VC, in parentheses is calculated as a percentage. Cells were also cultured at 5.6 mM glucose to determine drug toxicity. Toxicity is expressed as percent of dead cells, and listed as mean ± SEM for the number of experiments indicated in parentheses. Statistical significance of differences with the corresponding condition preincubated without glucose is calculated by unpaired Student's *t* testing, *P*; NS, not significant.

development of chemical (11–14), viral (28, 29), or autoimmune (30–32) diabetes. Certain antidiabetogenic effects are generated by direct interactions with the target pancreatic B cells. Thus, an increase in plasma glucose levels reduces the sensitivity of islet B cells to a subsequent alloxan exposure (13, 33), while the administration of nicotinamide protects the cells against streptozotocin (12) or against naturally occurring toxic agents in non-obese diabetic (NOD) mice (30). The underlying mechanisms of these protective effects are not yet fully understood but may involve the generation of those intracellular substances that are specifically depleted by a particular toxic agent, such as reducing equivalents after alloxan exposure (34) or NAD after streptozotocin treatment (12, 35, 36). It remains, however, completely unknown whether the target B cells actively participate in their own destruction—for example, by gearing defense mechanisms once they have undergone cellular injury of any kind. To assess the existence of such protective reactions in pancreatic B cells, an *in vitro* model system was developed for the identification of components that promote or prevent (non)-specific B-cell death. The method takes advantage of the availability of purified islet B and non-B cells for *in vitro* analysis (18–21). In the present report, this new experimental approach is employed to investigate whether the survival of drug- and complement-treated B cells can be varied by altering the environmental conditions between the moment of cellular attack and that of cell death.

t-Butylhydroperoxide, a potent oxidant in various cell types, was toxic to both islet B and non-B cells. In both islet cell populations, cell death was reduced when nicotinamide was added immediately after the initial *t*-butylhydroperoxide treatment. This inducible protection in toxin-treated cells suggests the existence of a reversible phase in the process of B-cell damage, a stage that has already been described in other cells and tissues (16). Culturing the *t*-butylhydroperoxide-treated cells in 20 mM glucose also enhanced the survival of islet B cells but not of glucagon-containing A cells, indicating the existence of a B-cell-specific glucose protection against cell injury. Such a protective effect may already have occurred in the basal culture medium containing 5.6 mM glucose, thus accounting for a lower *t*-butylhydroperoxide toxicity in islet B cells relative to non-B cells. Neither galactose nor fructose protected *t*-butylhydroperoxide exposed B cells, which suggests that the protective action of glucose is the result of its rapid metabolism in pancreatic B cells.

Glucose and nicotinamide also counteract the destruction of islet B cells that had been exposed to the diabetogenic agents alloxan and streptozotocin. Both poisons are known to selectively kill pancreatic B cells (37), which was, to our knowledge, not yet directly documented *in vitro*. In the present work, alloxan and streptozotocin were shown to exert a dose-dependent toxicity on insulin-containing B cells while failing to affect the survival of other islet cell types at the same concentrations. This B-cell selectivity may result from the capacity of the cells to rapidly incorporate both drugs with a short half-life (38–40) and from the exquisite sensitivity of the cells to the cytotoxic action of these drugs. The alloxan toxicity has been attributed to its direct interactions with islet protein kinase (41) and to its well-known ability to generate highly reactive oxygen radicals (42, 43). It is not yet clear whether the destructive effects of streptozotocin are also elicited by free radicals or whether they develop after alkylation of DNA bases (44–49). Differences in protective conditions have often been considered as arguments in favor of a different action mechanism of the two diabetogenic compounds. This was, for example, the case after *in vivo* experiments had demonstrated that pretreatment with glucose protected against alloxan but not against streptozotocin, while the prior administration of nicotinamide selectively reduced the toxicity of streptozotocin (12, 50, 51). The present *in vitro* experiments on purified B cells confirm these findings in that the addition of glucose prior to or during drug exposure reduced the cytotoxic effects of alloxan but not of streptozotocin, while the presence of nicotinamide during the same period counteracted only the streptozotocin-induced effects. However, when glucose or nicotinamide were also included in the medium immediately after the drug exposure, both compounds were found to protect against both alloxan and streptozotocin. That the alloxan-exposed B cells benefited from glucose protection in an earlier phase than streptozotocin-treated cells can be attributed to the fact that, within 3 min, alloxan elicited a cellular oxidation that was neutralized by glucose, whereas streptozotocin did not alter the cellular redox state within the first 15 min. Since streptozotocin may exhibit a longer lag time in its cytotoxic mechanism (47, 52), its oxidative effects could develop later than those of alloxan, which would explain the later occurrence of the glucose protection in the streptozotocin model. Rather than suggesting an action mechanism for alloxan and streptozotocin, the present data warn against the use of protection experiments for this purpose, especially if these studies are not extended over the period immediately following the cell aggression. The relative importance of the phase between the initial cell attack and the moment of irreversible cell damage is also illustrated by the finding that nicotinamide is most effective during this period; while this protective agent reduced cell death only by 25% when present before or during the 3-min streptozotocin exposure (data not shown), it exerts a much more potent protection against both alloxan and streptozotocin when added immediately after drug exposure. This observation is not necessarily in contradiction with reports on a marked antidiabetogenic action of nicotinamide injections administered prior to the drug (12, 35). It is indeed conceivable that the *in vivo* administration creates a sufficiently long elevation in nicotinamide levels to exert protective effects at a later stage.

Among the agents that have been implicated in the development of human diabetes, islet cell antibodies have been closely correlated with the clinical onset of the insulin-dependent form of the disease (53–56). In young recently diagnosed diabetics, circulating immunoglobulins have been detected that bind specifically to the surface of rat pancreatic B cells (24) and that can impair their secretory function *in vivo* and *in vitro* (57, 58). Certain of these antibodies also mediate a complement-dependent islet cell destruction (59), which appeared to be B-cell specific for the immunoglobulin fraction tested in this study. As with the three toxic agents previously discussed, the cells exposed to surface antibodies plus complement were also protected—be it to a minor extent—when cultured with nicotinamide and high glucose levels. The concept that target cells can counteract complement-mediated immunocytotoxicity has already been described for hepatoma cells (60) and may proceed via lipid-dependent membrane repair or internalization of afflicted membrane areas (61).

Besides offering the possibility of identifying components and mechanisms that lead to B-cell specific death, the *in vitro* model developed also permits an assessment of the role of pancreatic B cells in their own destruction process. It was thus shown that physiologic compounds can reduce the cellular susceptibility to a particular diabetogenic agent or to the destruction process in general. Particularly in the period immediately following exposure to the toxic agent, the target B cells could be stimulated to counteract the damage; the effectiveness of this cellular defense is likely to vary with the length of the reversible phase of the cellular injury. In studying four different modes of B-cell destruction, it was found that glucose and nicotinamide enhanced the survival of B cells treated with any of the four agents. Nicotinamide

could also protect islet non-B cells perhaps by restoration of the depleted NAD stores. Glucose-induced protection appeared to be specific for pancreatic B cells and may be mediated by reducing equivalents thought to neutralize the oxidizing effect of free radicals; glucose-dependent alterations in cellular calcium metabolism may also increase the resistance of pancreatic B cells, as they did in isolated hepatocytes (62). Regardless of the intracellular mechanism involved, the rapidity of glucose handling and recognition by pancreatic B cells may well explain its potent and B-cell specific protective action. The knowledge that glucose not only stimulates the division of pancreatic B cells (63) but also activates their defense mechanism raises the possibility that hyperglycemia may be beneficial during an early phase in the pathogenesis of diabetes. An inadequate B-cell response to glucose may, on the other hand, contribute to the progression of the disease. It is concluded that the development of insulin-dependent diabetes is not only determined by the severity of a B-cell-specific attack but also by the capacity of the target B cells to counteract the injuring process.

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