Dietary Exposure to 2-Aminoanthracene Induces Morphological and Immunocytochemical Changes in Pancreatic Tissues of Fisher-344 Rats

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Toxic chemicals ingested as the result of environmental exposures or other risk factors such as cigarette smoking may increase the risk of developing cancer and other diseases such as diabetes. 2-Aminoanthracene (2-AA) was investigated to determine toxic effects of chronic dietary exposure upon major organ systems including the pancreas. Fisher-344 rats were fed 2-AA (50–100 mg/kg of diet) and euthanized at 14, 30, 63, and 80 days. Growth, tissue histological, immunocytochemical, and clinical pathological end points were examined at each time point. Significantly elevated plasma glucose and glycated hemoglobins and reduced serum protein levels were recognized after 80 days of feeding (100 mg/kg of diet 2-AA group). Similar results were observed in rats exposed to 75 mg/kg of diet but appeared to be absent in the 50-mg/kg group. An unexpected pattern of responses suggestive of diabetic sequelae was observed in a glucose tolerance test conducted during the seventh week. After 63 and 80 days, large cytoplasmic vacuoles in islet cells were observed by light microscopy. In addition, the immunocytochemical study demonstrated beta cell insulin insufficiency at 63 and 80 days. No inflammatory infiltration of the islets was observed. These findings suggest that depletion of secretory granules occurred in the beta cells. Necrotic changes occurred in the acinar cells of the pancreas with increasing duration and dose of 2-AA. The cytological, immunocytochemical, and histological results demonstrate that chronic dietary exposure to 2-amino anthracene alters the endocrine and exocrine pancreas cellular morphology and induces diabetic-like symptoms in the Fisher-344 rat.

Key Words: blood glucose; 2-aminoanthracene; immunoenzyme; insulin; islets of Langerhans; rats.

The rat pancreas has frequently been used as an animal model to study changes in islet cells in different pathological conditions, such as diabetes mellitus and pancreatic tumors (Elayat et al., 1995). The islets of Langerhans synthesize, store, and secrete a variety of polypeptide hormones and form the endocrine portion of the pancreas. The endocrine portion of the pancreas consists of the individual islets scattered throughout the acinar parenchyma. It has been hypothesized that the arrangement of cell types within the islets and resulting cell-to-cell communication may have physiologic significance in the control of glucose homeostasis (Elayat et al., 1995; Orci, 1982; Orci and Unger, 1976). In insulin-dependent diabetes in animals and man there is a marked disruption of the normal histologic relationships (McEvoy and Hegre, 1977). Insulin-dependent diabetes mellitus (IDDM) (Type I) results from specific destruction of the pancreatic islet insulin-secreting beta cells (Rabinovitch et al., 1996b; Stefan et al., 1982).

For decades a number of other toxic agents have been used to induce diabetes mellitus in experimental animals. Streptozotocin and alloxan are the best known of these agents that exert their adverse effects via selective necrotic changes in the islets of Langerhans resulting from the production of highly reactive oxygen radicals. In humans, diabetes mellitus represents a heterogeneous disorder in both its pathogenesis and etiology, and the possibility of an association between environmental exposure to toxic agents and the development of this disease appears reasonable.

Aromatic amines are synthetic and naturally occurring compounds, which are well known as occupational carcinogens that are used in the manufacture of dyes, drugs, inks, plastics, and agricultural chemicals (Cartwright, 1983; Gorrod and Manson, 1986; Kriek, 1979). Aromatic amines are also used as rubber antioxidants, curing agents in the synthesis of epoxy resins and polyurethanes, and are present in road tars, and synthetic fuels (Garner et al., 1984; Gorrod and Manson, 1986; Snyderwine et al., 1992). The benchmark toxicant, 2-aminoanthracene (2-AA), is a model aromatic amine since it is a relatively potent direct-acting carcinogen in animal models, and an inducer of mutations in both eukaryotic and prokaryotic cells (Arcos and Argus, 1974; Carriere et al., 1992; Fu et al., 1986; Hallstrom et al., 1981; Shear, 1983; Shubik et al., 1960; Zhu et al., 1995). Tobacco smoking and cooked foods are non-occupational sources of amines, and humans are frequently
exposed to arylamines in mainstream and sidestream cigarette smoke and charbroiled proteinaceous foods (Butler et al., 1989; Vineis, 1992, 1994). The present study was undertaken to examine the effects of chronic dietary exposure to 2-aminoanthracene in the Fisher-344 rat, including toxicity and changes in the major organs, including the pancreas by means of histological and clinical chemistry. Immunocytochemical methods were also applied for the study of cytoplasmic localization of insulin, c-Ras and c-Myc. The results indicate a mechanistic role for 2-AA in the induction of diabetic-like symptomology following chronic dietary exposure to 2-AA.

MATERIALS AND METHODS

Chemicals. 2-AA (98–99% pure) was obtained from Aldrich Chemical Co., Inc (Milwaukee, WI). Chemicals for the diet formulation were purified, high nitrogen casein (ICN, Costa Mesa, CA); corn starch (ICN); alphcel (ICN); dextrinized corn starch (ICN); tert-butylated hydroquinone (ICN); l-cystine (ICN); soybean oil (ICN); AIN-93M mineral mix (Harlan-Lab, Madison, WI); AIN-93M vitamin mix (Harlan-Teklad); and choline bitartrate (Harlan-Teklad).

Diet. A basal, semi-purified powdered rodent diet (AIN-93M) was prepared according to the formulation recommendations of the American Institute of Nutrition (1993). In brief, dry dietary ingredients were mixed in a commercial floor model mixer, after which the prescribed amount of soybean oil (ICN) was added and mixing continued until a homogeneous mixture was obtained. The basal diet was prepared in 5 kg batches once a month and stored at −20°C. The unadulterated basal diet served as the control diet. 2-AA was incorporated into three separate batches of the basal diet powder at 50, 75, and 100 mg/kg dry weight, respectively. Pilot studies (data not shown) established that the concentrations of 2-AA used in the present study either were nonlethal or had the projected potential of causing only limited lethality (<10%) following chronic administration through the diet. Incorporation of the test compound into the basal diet was accomplished by blending on an electric bottle-roller bar assembly for 3 h at room temperature in amber glass jars in the dark.

Animal treatment. Animals were fed on a defined diet with water ad libitum. 2-AA was administered to the rats through one of the three adulterated diets. A single control diet was used throughout each experiment. The control and three adulterated diets (50, 75, and 100 mg 2-AA/kg of diet) corresponded to a dosage of approximately 0, 5, 7.5, and 10 mg/kg of body weight, respectively, per day based upon an average rodent food consumption pattern of 10% body weight per day. Fresh diet was provided to each animal daily and individual rats were assessed daily for measurements of dietary intakes and weekly measurements of body weights. Daily observations of the rats were made during feeding and/or cage cleaning/bedding changes and recorded in a log. Changes in feeding habits, urination and feces production, general activity levels, and excitability were all noted and recorded. At weeks 2, 4, and 9 of the exposure period of Experiment 1, five 2-AA−exposed rats (100 mg/kg-diet group) and five control rats (0 mg/kg-diet group) were anesthetized by carbon dioxide inhalation, blood samples were taken from the abdominal vena cava distal to the pancreas and the animals were euthanatized by exsanguination. The pancreas were examined and excised at autopsy. At the 11th week of exposure of Experiment 2, seven 2-AA−exposed rats (100 mg/kg-diet group), five 2-AA−exposed rats (75 mg/kg-diet group), five 2-AA−exposed rats (50 mg/kg-diet group), and 11 control rats (0 mg/kg-diet group) were similarly sampled and euthanatized by exsanguination.

Glucose tolerance test. A glucose tolerance test was performed on the ninth week in overnight fasting rats (three per experimental group) by oral administration of a 40% aqueous solution of glucose (Sigma, St Louis, MO) at a dosage of 4 g/kg body weight by gavage. Rats were anesthetized by isoflurane inhalation at each blood collection time point. Blood samples were taken by alternating retroorbital plexus sites (1 ml per collection per rat) at 0, 30, 60, 120, and 180 min after the oral glucose dose for the measurement of plasma glucose and insulin levels. Plasma glucose was measured spectrophotometrically using the glucose oxidase method (Kadish et al., 1968). Insulin levels were determined by radioimmunoassay using a commercially available 125Iodine kit for human insulin (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA).

Clinical chemistry. Serum was prepared by allowing the blood sample collected in gel-clot tubes to clot at room temperature for 15–30 min. The sample was then centrifuged for 10 min at 2000 × g. Serum chemistry profiles were obtained by automated procedures (Olympus Reply, Olympus America, Inc, Lake Success, NY) and included determinations of blood urea nitrogen (BUN), creatinine, and total protein. Plasma glucose was measured spectrophotometrically using the glucose oxidase method (Kadish et al., 1968). Insulin levels were determined by radioimmunoassay using a commercially available 125Iodine kit for human insulin (Coat-A-Count, Diagnostic Products Corp.). Glycated hemoglobins were measured using a commercially available test kit (Isolab, Inc, Akron, OH).

Tissue preparation. For histological light microscopy, the pancreata of the rats were immediately excised and tissues were fixed in a solution of neutral-buffered 10% formalin for 24 h. Sections of tissues were dehydrated through increasing concentrations of ethanol, and the fixed sections were embedded in paraffin. The paraffin blocks were sectioned (5 mm) and the sections were placed on albumin-coated slides, deparaffinized, hydrated through graded xylene and alcohol, processed, and stained with hematoxylin-eosin. Sections were then cleared and mounted with nonaqueous cover slip resin (Permount, Fisher Scientific, Cincinnati, OH). Stained sections from each rat were used for morphometric analysis.

Immunocytochemistry for light microscopy. Tissue sections on albumin-coated slides were deparaffinized, hydrated through decreasing concentrations of ethyl alcohol, rinsed in deionized water, and finally rinsed in PBS (pH 7.4). Tissue sections were washed in methanol with 0.5% hydrogen peroxide at room temperature for 30 min to quench endogenous peroxide activity. Antigenic determinants masked by formalin-fixation and paraffin-embedding were freed by pretreatment of tissue sections with 0.1% pepsin in 0.01 N hydrochloric acid (pH 2.3) at room temperature for 15 min. Enzyme digestion was terminated by repeated washing in PBS and finally by washing in PBS with 0.1% Tween-20. The prepared tissue sections were then subjected to an avidin biotin complex (ABC) treatment for immunocytochemical examination (Hsu et al., 1981). In each case, the diluting buffer was phosphate buffered saline (pH 7.4).

Insulin secretory B cells were visualized in rat pancreata by employing methods supplied in the Vectastain ABC kit (Vector Laboratories, Burlingame,
CA). Guinea pig antiporcine insulin (Dako Corp., Carpinteria, CA) and normal guinea pig serum were applied to the tissues as the primary antiserum (optimum dilution 1:150) and incubated for 45 min at room temperature. Subsequently, the biotinylated rabbit antiguinea pig immunoglobulin (dilution of 1:200) was employed as the secondary antiserum (Vector Laboratories). The chromogen substrate used for visualization of insulin was amino ethylcarbazole (AEC) and counter staining was with Meyer’s hematoxylin. Sections were cleared and mounted with an aqueous cover slip resin (Aquamount, Baxter Laboratories). The pancreatic localization and distribution of the oncogenes, ras, and c-myc were visualized by the use of an ABC kit (Vector Laboratories). The sequence of antibodies applied was (1) a mouse monoclonal antibody for ras diluted at 2.5 mg/ml or a rabbit polyclonal antibody for myc at a dilution of 1:50 and (2) an appropriate biotinylated secondary antibody directed against the host for the primary antibody (as directed in the Vectastain kit). The clone pan-ras10 anti-ras (Oncogene Research Products, Cambridge, MA) was a purified mouse monoclonal to an undetermined epitope of the Ras protein common to K-ras, H-ras, and N-ras. Pan-ras (Ab-3) was shown to react with the ras p21 proteins of human, mouse, and rat origin by Western blotting, immunoprecipitation, immunofluorescence, and immunohistochemistry. The anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 1-262 mapping at the amino terminus of c-Myc (N-262) of human origin and was shown to react with c-Myc p67 of mouse, rat, and human origin by Western blotting, immunoprecipitation, and immunohistochemistry. The reaction products were visualized using the AEC chromogen substrate and counter staining with Meyer’s hematoxylin. Sections were cleared and mounted with an aqueous mounting medium (Aquamount, Baxter Laboratories Boronia Victoria, Australia).

**Statistical analysis.** Statistical analyses were performed using PC-SAS (SAS 6.04; SAS Institute, Cary, NC). Data were expressed as mean ± SEM, unless otherwise indicated. Data were analyzed by analysis of variance. Significant differences among treatment groups were tested by the Tukey Studentized post hoc multiple comparisons test (α = 0.05), when appropriate. The univariate and frequency procedures were employed to obtain information about the distribution of these parameters among the different treatment groups. Weight and intake data were analyzed using a repeated measures variance about the distribution of these parameters among the different treatment groups.

**RESULTS**

Body weight gain and dietary intake levels were compared between 2-AA–treated and untreated rats during the 80 days of the experiment. Significant decreases in body weight gain were observed in rats fed diets adulterated by the addition of 2-AA at 75 mg/kg and 100 mg/kg of diet. As shown in Figure 1, a significant decrease in body weight gain compared with the untreated control animals was noted in the group of rats exposed to 2-AA at 100 mg/kg of diet when compared with control animals. A similar trend was shown in the group of rats exposed to 2-AA at 75 mg/kg of diet, whose body weight gain was significantly lower at the end of week 2 when compared with the respective control group (Fig. 1). The departure in weight gain compared with control animals remained significant in both these groups of 2-AA treatments for the remainder of the experimental period. On the other hand, no significant departure from control animals in body weight gain was observed among rats treated with 2-AA (50 mg/kg of diet). Similarly, as shown in Figure 1, a significant decrease in dietary intake was observed for animals exposed to 2-AA (100 mg/kg of diet) at week 2 of the experiment and anorexia persisted in this group of animals throughout the remainder of the study. In contrast to the significant decrease in body weight gain observed in rats exposed to 2-AA (75 mg/kg of diet), a significant decline in dietary intake was observed in these animals for weeks 7 and 8 of the study only (Fig. 2), suggesting

**FIG. 1.** The effects of 2-AA on animal body weight. Each data point represents the average group weekly body weight of 10 male F-344 rats exposed at 100 mg/kg of diet 2-AA or untreated (0 mg/kg of diet) (Experiment 1), or five rats exposed at 75 mg/kg of diet or 50 mg/kg of diet 2-AA and untreated (0 mg/kg of diet) (Experiment 2). All points after week 2 for the 75 mg/kg of diet and 100 mg/kg-diet exposure groups are significantly lower than controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
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<tbody>
<tr>
<td>Glucose (mg/dl)</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Untreated</td>
<td>100 ± 5a</td>
<td>187 ± 69b</td>
<td>191 ± 5b</td>
<td>219 ± 80b</td>
<td>212 ± 40b</td>
</tr>
<tr>
<td>Treated</td>
<td>97 ± 14a</td>
<td>390 ± 141a</td>
<td>410 ± 138a</td>
<td>493 ± 215a</td>
<td>422 ± 109a</td>
</tr>
<tr>
<td>Insulin (mU/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Untreated</td>
<td>169 ± 33a</td>
<td>282 ± 172a</td>
<td>230 ± 60a</td>
<td>201 ± 80a</td>
<td>176 ± 68a</td>
</tr>
<tr>
<td>Treated</td>
<td>185 ± 44a</td>
<td>331 ± 155a</td>
<td>268 ± 112a</td>
<td>209 ± 90a</td>
<td>268 ± 142a</td>
</tr>
</tbody>
</table>

*Note.* Each data represent the mean (± SEM) of five animals. Means with the same letters are not statistically different from untreated animals, *p* < 0.05.
the presence of cachexia in spite of normal dietary levels of intake. The rats exposed to lowest level of 2-AA (50 mg/kg of diet) did not significantly differ from control animals in dietary intake at any time point during the course of this study. Qualitative daily cage side observations of exposed as compared with control rats that were made indicated the presence of polydipsia, diuresis, cachexia, and perhaps symptoms of neuropathy in the treated rat groups but not in controls.

Plasma glucose levels were compared between 2-AA–treated and untreated control rats at termination of the experiment. As shown in Figure 3, the levels of plasma glucose at the termination point of the experiment were significantly elevated by dietary exposure to 2-AA at 100 mg/kg and 75 mg/kg, respectively. Plasma glucose values were 475 and 636 for nonfasted rats exposed to 2-AA at 100 mg/kg and 75 mg/kg of diet, respectively. The reported range of normal male rat plasma glucose values is 108–224 ± 28.6, while in streptozotocin-induced diabetic rats plasma glucose values ranged from 418 to 802 ± 108 (Alder et al., 1992; Hosgood, 1996).

Glucose in plasma at elevated concentrations can attach to a variety of proteins nonenzymatically (Brodrick et al., 1993). For example, glucose can interact with red blood cell hemoglobin to form stable covalent bonds, a reaction that occurs in proportion to blood glucose concentrations (Bunn, 1981). Glycated hemoglobin, therefore, provides an accurate measure of serum glucose levels, independent of diet and fasting status. Percentage of glycated hemoglobin (Fig. 4) was elevated by exposure to 2-AA at 100 mg/kg of diet and 75 mg/kg of diet, respectively. The normal percentage of glycated hemoglobins in rats ranges from 3.1 to 4.7 ± 0.38, while the range of percentage of glycated hemoglobins is 7.9–10.8 ± 0.95 in a streptozotocin-induced diabetic rats (DeTata et al., 1996; Hosgood, 1996). Mean percentages of glycated hemoglobin were 13.0 and 14.5 in rats exposed to 2-AA at 100 mg/kg and 75 mg/kg of diet, respectively.

Assessment of the effects of a chemical on kidney function can be performed by evaluating serum chemistry levels of BUN, serum protein levels, and creatinine. Values for these clinical chemistry parameters are shown in Figures 5 and 6. As shown in Figure 5, BUN was significantly elevated by dietary exposure to 2-AA at both 100 and 75 mg/kg of diet, when compared with the respective control groups, while serum protein levels were decreased at these two dose levels as shown in Figure 6. Creatinine levels were unaffected by 2-AA exposure levels. Chemically induced increases in BUN may not necessarily reflect kidney damage, but may be secondary to dehydration and/or protein catabolism, both of which are symptoms of diabetes.

The results of the glucose tolerance test are shown in Table 1. Plasma glucose levels of 2-AA–treated rats were significantly higher in all samples taken at time intervals following oral glucose administration, as compared with those of untreated rats. In the former cases, moderate increase in glucose levels was shown in the plasma at 30 and 120 min, but no obvious change could be found in the untreated animals. In contrast, insulin levels of 2-AA–treated rats were remarkably similar to those of untreated rats in all samples taken at the times shown in Table 1.

Histological examination by light microscopy could not detect a change in the islet cells of the pancreas or in the architecture of the exocrine pancreas during 14 and 30 days of the exposure to 2-AA at 100 mg/kg of diet, nor were histologic changes observed in the endocrine or exocrine pancreas of rats fed 50 mg/kg of 2-AA–adulterated diet, as shown in Figure 7A. At 63 days of treatment, a few cells in almost all of the islets examined had some cytoplasmic vacuoles, which were faintly and homogeneously stained with eosin, as shown in Figure 7B. In animals treated for longer than 63 days with 2-AA–adulterated diets at 100 mg/kg and 75 mg/kg of diet, the cytoplasmic vacuoles were observable in most cells, except for the few remaining cells on the periphery of the islets. The size of these vacuoles varied to the extent that some of the vacuoles were large enough to occupy a major portion of the cytoplasm and displace the nuclei as shown in Figures 7C and 7D. Gross morphological changes in the exocrine pancreas of these animals were observed as well. Membrane ghosts of acinar and islet cells were observed throughout much of the pancreas of rats exposed to 100 mg/kg of diet and 75 mg/kg of diet of 2-AA–adulterated diets for 80 days. There was no observable infiltration of lymphocytes or macrophages in the pancreas of control or treated rats suggestive of any inflammatory response.

Using guinea pig antiporcine insulin as primary antiserum, clear positive reaction products to insulin were observed in
almost all of the islet cells of pancreas, except for peripheral cells, from untreated animals. A similar staining pattern was also observed in the pancreas of rats treated for 14 and 30 days with 2-AA at 100 mg/kg of diet of adulterated diet and from rats treated with 2-AA at 50 mg/kg of adulterated diet, as shown in Figures 8A–D. In the case of 2-AA exposure for longer than 63 days, as shown in Figures 8E and 8F, the staining intensity became fainter with increased duration and dose of 2-AA.

Histological examination by light microscopy did not detect differences in the positive reaction products to ras oncogene protein in the pancreas of 2-AA–treated rats regardless of dose or duration, and no discerning differences in Ras reaction products were observed between the pancreases of untreated versus treated rats. A very faint, nonspecific stain was observed in the cytoplasm of almost all acinar cells whether the pancreas originated from an untreated or 2-AA–treated rat. Since observations failed to detect heightened expression of ras in treated animals, the faint staining was deemed constitutive expression. A distinctive pattern of peripheral islet-cell staining of reaction products to the c-myc oncogene protein was detected in the islets of pancreas derived from untreated rats. This pattern of staining was also common in the pancreas of rats treated for less than 63 days, and in the pancreas of rats treated for 80 days with 2-AA at 50 mg/kg of diet, as shown in Figure 9A. As shown in Figure 9B, the pancreas of rats exposed for greater than 63 days to 2-AA at 75 mg/kg or 100 mg/kg of diet displayed a different pattern of staining for the myc oncogene protein compared with untreated animals. Unlike the peripheral islet-cell–confined staining pattern observed in untreated rats and those treated with 2-AA for less than 63 days, clear positive reaction products to c-myc were observed in many of the acinar cells. The pattern of most intense staining for c-myc reaction products was observed in the acinar cells surrounding islets that showed the greatest amount of toxic insult, e.g., large cytoplasmic vacuoles. The observed pattern of acinar cell staining was detected only in the pancreas of rats exposed for greater than 63 days of 2-AA treatment and fed 2-AA–adulterated diets at 100 mg/kg or 75 mg/kg of diet.

**FIG. 3.** The level of plasma glucose was measured in treated and untreated nonfasted rats at termination. Each bar represents the treatment group mean (± SEM) of five rats treated and of four rats untreated, except for treated (100 mg/kg of diet 2-AA) and untreated (0 mg/kg of diet) groups (Experiment 1) which are the mean of seven rats.
DISCUSSION

One of the first signals that alerts investigators to the possible presence of diabetes mellitus is hyperglycemia, and hyperglycemia is often one of the first signals that suggests a chemical compound is a pancreatic β-cell toxin (Porte and Schwartz, 1996). In humans, clinical manifestations of diabetes mellitus include hyperglycemia (serum glucose > 180 mg/dl), diuresis, proteinuria, glucosuria, polydipsia, cachexia, elevated BUN, dehydration, and neuropathy. The observational, serological, and pathological results presented in these experiments support the findings that chronic dietary exposure to 2-AA at 100 mg/kg of diet or 75 mg/kg of diet for 80 days induces diabetes in the F-344 rat model. The elevated serum glucose and BUN levels, percentage of glycated hemoglobins, the low serum protein levels, the depressed dietary intake, and weight gain are indicative of diabetes mellitus. The glucose tolerance test, administered to rats at 62 days of dietary exposure to 2-AA, provides further support for this finding and serves as a useful tool for evaluating endocrine pancreatic function. A rapid rise in blood glucose levels was observed immediately following ingestion of the glucose load in treated and untreated rats. However, unlike the steady decline in blood glucose levels at subsequent time points as observed in untreated rats, the blood glucose levels in rats exposed to the toxic effects of 2-AA remained elevated and were still significantly elevated 3 h after the glucose load was administered. The significantly elevated levels of blood glucose in the presence of normal insulin levels is suggestive of insulin resistance (a subnormal biological response to insulin), and suggests that the insulin secreted by the β-cells is either nonfunctional or poorly functional in the 2-AA–treated rats.

Immunocytochemical evaluation with light microscopy and using porcine anti-insulin as the primary antiserum, demonstrated that insulin was detected in the β-cells of both 2-AA–treated and untreated animals, however, the pattern of staining intensity became weaker with increasing dose and duration of 2-AA exposure. Only faintly stained reaction products were detected in the β-cells of rats exposed to 2-AA at 100 mg/kg and 75 mg/kg of diet for 80 days, suggesting that insulin stores were almost entirely depleted. Additionally, the cytoplasmic
levels has a reported cross-reactivity with pro-insulin of 45%,
the total insulin immunoreactivity measured with the anti-
insulin antibody represents the sum of "true" insulin plus the
pro-insulin and any pro-insulin–conversion intermediates pres-
ent. This systematic overestimation of the insulin concentration
is relatively minor in normal animals whose pro-insulin and
intermediates are typically low, however, in cases of impaired
glucose metabolism, the pro-insulin forms may be disproport-
ionately higher (Heaton et al., 1988). Pro-insulin could be
released from the rough endoplasmic reticulum of damaged
cells during disintegration and a portion of the insulin detected
in the blood of exposed rats may have been in the nonfunctional
pro-insulin form (Morgan and Lazarow, 1963).

Diabetes often manifests itself in phases, and insulin and
glucose levels fluctuate as these phases of the disease progress.
Plasma insulin levels have been shown to decrease during the
early phase of hyperglycemia in alloxan-induced diabetic mice
and then to increase markedly to greater than normal levels
during a hypoglycemia phase. After the establishment of overt
diabetes, plasma insulin levels returned to levels below the
controls and became very low or immeasurable within the
drive of a few weeks (Rerup, 1970). In the present study,
serological and histological evaluations indicate that prior to
63 days of 2-AA dietary exposure, there is no evidence of dia-
abetes or pancreatic toxicity in exposed rats. Histological ob-
servations of the pancreases of rats exposed for 63 days indicate
the presence of cytoplasmic vacuoles in the β-cells of many of
the islets, yet nonfasting serum glucose values were normal.
The rats, at this point of 2-AA pancreatic toxicity (63 days),
may have entered the hypoglycemic phase of the diabetic
disease process.

Some important cytological and immunocytochemical find-
ings were obtained in this study that might facilitate the
elucidation of the mechanism by which 2-AA induces pancre-
atic toxicity. The mechanisms through which chemical-induced
β-cell toxicities are mediated remain controversial; however,
two main theories are emerging (Malaisse, 1986). The first
mechanism of chemical-induced pancreatic islet-cell toxicity,
for which alloxan is an archetypical agent, is disruption of the
plasma membrane integrity. Alloxan causes increases in β-cell
permeability due to binding of sulfhydryl groups on the plasma
membrane. Degenerative changes ensue due to the production
of oxygen radical species and the heightened sensitivity of
the β-cell to reactive oxygen species (Pieper et al., 1996a,b). The histological changes in islets
after alloxan intoxication have been reported and indicate that
the β-cell nucleus undergoes karyolysis, the cytoplasm com-
ponents disintegrate, the cell boundaries disappear, and finally
a mass of debris containing fragments of nuclei appear during
the final phases of the necrotic process. Acinar cells are only
slightly affected, and there is a complete absence of a mono-
nuclear cell response. No other histological changes are
encountered (Cooperstein and Watkins, 1981). A second
mechanism of chemical-induced pancreatic islet-cell toxicity,

vacuoles showed no evidence of staining, indicating a loss of
β-cell granules. Although the detection of normal serum insulin
levels for the glucose tolerance test and the histological
observations of depleted β-cell insulin stores in rats exposed
to 2-AA at 100 mg/kg of diet and 75 mg/kg of diet seem
contradictory, the data can be reconciled. While the measure-
ment of the peripheral serum insulin concentrations provides
valuable information, there are significant limitations inherent
in estimates of pancreatic insulin secretion rates based entirely
 upon peripheral insulin concentrations (Polonsky, 1995). Ins-
ulin is secreted directly into the portal vein rather than into the
peripheral circulation, and the liver is the major site of insulin
clearance and typically extracts approximately 50% of the
hormone on first pass (Jaspan and Polonsky, 1982). Fractional
hepatic insulin extraction may vary under different physiolog-
ical conditions, especially conditions that alter insulin concen-
trations (Farber et al., 1981). Another limitation of the glucose
tolerance test is the radioimmunoassay kit used to measure
insulin. Virtually all the commercial polyclonal anti-insulin
antibodies used to measure insulin have a high degree of cross-
reactivity with pro-insulin and with pro-insulin–conversion
intermediates. Since the anti-insulin antibody provided in
radioimmunoassay kit used in this study to measure insulin

for which cyproheptadine is an archetypical agent, is the inhibition of the transfer of insulin precursors from the rough endoplasmic reticulum to the Golgi apparatus. Cyproheptadine toxicity results in the accumulation of the synthesized precursors in the rough endoplasmic reticulum (Hanai, 1984). The effects of antihistaminic drugs are characterized histologically by a depletion of secretory granules and formation of large cytoplasmic vacuoles in the \( \beta \)-cells, vesiculation of the rough endoplasmic reticulum, while necrosis is absent (Hanai, 1984; Longnecker et al., 1972; Rerup, 1970). The morphologic and biochemical changes are reversible upon termination of exposure and the pancreotoxicity is species-specific, observed only in the rat. Also, the toxic effects of the drug were greater after oral exposure than after ip injection.

The cytological, immunocytochemical, and histological results presented in this study indicate that 2-AA-induced pancreatic toxicity followed neither of these reported mechanisms precisely, but rather characteristic changes of both mechanisms were observed, suggesting that 2-AA may exert its effects through an independent mechanism which is as yet undefined. As with alloxan toxicity, histological changes resulting from 2-AA exposure included the disintegration of cytoplasm of \( \beta \)-cells, the disappearance of cell boundaries and that was characterized by our pathologist as necrosis but which could also be apoptosis. Apoptosis is often observed in response to chemical carcinogens in the pancreas and is typically associated with activation of cell death receptors belonging to the tumor-necrosis factor (TNF) superfamily of receptors (Itoh et al., 1991; Mathias et al., 2001; Suda et al., 1993; Westphal and Kalthoff, 2003) and also with \( c \)-myc expression observed here. While we did not assess the activation of these markers directly in these tissues, in our tumor studies we did observe strong activation of TNF-\( \alpha \) in animals exposed in parallel with these (Boudreau et al., 2001).

Also in other species, we have observed the activation of other apoptosis-related gene products such as p53 and proliferating cell nuclear antigen in response to exposure to 2-AA (Lentz et al., 2003, 2006). A free radical mechanism would also explain the apparent necrosis/apoptosis observed in the exocrine pancreas. The metabolism of 2-AA is thought to proceed via hepatic N-oxidation, followed by acetylation and, since the acetyl function is a good leaving group, an aryl nitrenium ion group may be formed during, prior to, or after uptake of this compound by the pancreas. The nitrenium ion appears to be the ultimate electrophilic metabolite that binds DNA (Means et al., 2003; Rodrigues et al., 1993). Studies have shown that the N-acetylase activity in the liver closely correlates with the activity of that enzyme in the pancreas (Martell and Weber, 1993).

Furthermore, epidemiological studies indicated a prevalence of the rapid acetylator phenotype among IDDM patients. The F344 rat also expresses a rapid acetylator phenotype (Burrows et al., 1978).

On the other hand, as observed in the cyproheptadine-type toxicity, degranulation and the formation of large cytoplasmic vacuoles...
vacuoles were observed in the insulin-secreting β-cells of the pancreas of 2-AA–treated animals. Fortuitously, the rat was the study model chosen and ingestion was the route of exposure in this study. These may have been contributing factors to 2-AA–induced pancreatic toxicity. Immunocytochemical evaluation with light microscopy and using porcine anti-insulin as the primary antiserum demonstrated that insulin was detected in the β-cells of both 2-AA–treated and untreated animals, and the pattern of staining intensity became weaker with increasing dose and duration of 2-AA exposure. Additionally, the cytoplasmic vacuoles were devoid of stain, indicating a loss of beta-cell granules. These results suggest that the mechanism of beta-cell vacuolization by 2-AA does not originate in the rough endoplasmic reticulum since the cytoplasm did not stain positive, but rather suggests that cytoplasmic vacuolization may be caused by hydropic degeneration. Furthermore, unlike either alloxan- or cyproheptadine-type toxicity, vacuoles were observed in the pancreatic acinar cells of rats exposed to 2-AA for greater than 63 days at both 100 mg/kg of diet and 75 mg/kg of diet.

Several lines of evidence indicate that some of the known oncogenes exist in rat and are expressed under a variety of physiological conditions, including regeneration and neoplasia (Richmond et al., 1988). Indeed, increases in expression of growth control genes in response to cell loss or toxicity have been characterized in many organ systems (Roberts et al., 1997; Sprankle et al., 1996). To investigate the possibility that oncogenes serve as signals indicative of some aspects of 2-AA pancreatic toxicity and that the detection of these proteins in specific regions of the pancreas may aide in elucidating the toxic mechanism of 2-AA, the expression of ras and c-myc were examined. In untreated and treated rat pancreas tissues,
the expression of ras was near the threshold of detection in acinar cells and islet cells alike. Only a few isolated non-parenchymal cells along capillaries showed a distinct signal. In untreated rat pancreas tissues, expression of c-myc was detectable at low level in most peripheral islet cells, assumed to represent alpha and delta cells. A similar low level and intensity of staining was observed in 2-AA–treated rat pancreas tissues in 14-day, 30-day groups at all doses tested, and in the low-dose (50 mg/kg of diet) group exposed for 80 days. Both the pattern and intensity of c-myc reaction products were altered by 2-AA exposure at 100 mg/kg of diet and 75 mg/kg of diet for 80 days. Grains of deep intensity were predominantly localized over the acinar cell cytoplasm. A low level of c-myc reaction products was also observed in the islet periphery cells as in the islet cells of the untreated rats. The exocrine pancreas is under both short- and long-term neuronal and hormonal control. Pancreatic short-term regulation consists mainly of the stimulation of secretion due to food digestion, while long-term regulation consists of the regulation of pancreatic mass and alterations in the content of digestive enzymes according to the needs of the organism (Scaglia et al., 1997). It has been shown that nuclear oncogenes function as mediators coupling short-term signals, which are rapidly elicited by cell surface stimulation, to long-term responses, such as growth and differentiation, by regulating the expression of specific genes (Lu and Logsdon, 1992). The enhanced expression of c-myc, an immediate early gene, and the lack of enhanced expression of ras, is consistent with the role of c-myc in cell proliferation and suggestive of events occurring within the pancreas aimed at restoring its architectural integrity (Farquharson et al., 1992; Hasmall et al., 1997). These results imply that the myc oncogene may function in response to toxic insult of the pancreas and may play a role in the mechanism of 2-AA–induced pancreatic toxicity. However, the fact that the toxicity of 2-AA may involve extra-pancreatic factors cannot be eliminated. Additional studies are needed to elucidate the mechanism of 2-AA–induced pancreatic toxicity and the potential role of free radical mechanisms in this process. Further, analysis of gene expression patterns in response to chronic dietary exposure to 2-AA as a function of dose and time need to be conducted. These are the subjects of current studies being conducted in our laboratories.

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