A GLYCONUTRITIONAL MIXTURE (AMBROTOSE®) PROVIDES SOME AMELIORATION TO MICE WITH COXSACKIEVIRUS-INDUCED PANCREATITIS

C. Gauntt^{1*}, D. Busbee², H.J. Wood¹, S. Reyna¹, R. Barhoumi², R. Burghardt², W. McAnalley³ and H.R. McDaniel³

¹ Department of Microbiology, Mail Code 7758, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900.

² Department of Anatomy and Public Health, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4458.

³ Research and Development (W.M.) or Medical (H.R.M.) Divisions, Mannatech, Inc., Coppell, TX 75019

ABSTRACT

Challenge of adolescent male CD-1 mice with a coxsackievirus B3 (CVB3) strain (CVB_{3m}) induces mild to severe destruction of pancreatic acinar cells, but causes no deaths and does not induce hyperglycemia. A weekly parenteral (intraperitoneal) administration of a glyconutritional mixture (Ambrotose®) to virus-challenged mice was assessed to determine if there were any benefits to recovery over an eight month period. Virus-challenged mice showed a significant weight loss over the initial five weeks of the experiment, but injection of Ambrotose® to similar virus-challenged mice restored the total body weight to levels found in normal mice. Normal mice given Ambrotose® exhibited a small weight gain. Mice given Ambrotose® showed reduced severity of pancreatitis, as evidenced by significant reductions in percentages of pancreatic acinar cells destroyed and proportion of sections of pancreata with destroyed acinar cells, compared to virus control-mice not injected with Ambrotose®. Statistical analyses of the extent of acinar cell pathology in all virus-challenged mice showed that Ambrotose® contributed significantly to recovery of the acinar cell population in virus-inoculated mice. Anti-viral antibody titers were not affected by Ambrotose® injections. One potential mechanism to explain the benefits derived from Ambrotose® injections came from studies of antioxidant levels of glutathione in splenic macrophages/monocytes. Whereas CVB3 challenge of mice reduced glutathione levels in the latter cells, Ambrotose® injections to virus-challenged mice restored glutathione levels to those found in normal mice. In summary, most but not all mice derived benefits from Ambrotose® injections, i.e. a reduction in pathology in the pancreas and restored levels of the antioxidant glutathione in macrophages/monocytes. Higher doses of Ambrotose® could provide greater benefits for more mice, a study for the future.

INTRODUCTION

The coxsackieviruses of group B (CVB) have long been associated with pancreatitis and some cases of insulin-dependent diabetes mellitus (IDDM; Pallansch, 1988; Melnick, 1997). Between 40 and 80 percent of adults in the U.S. have been infected with one of the five serotypes (of six total) of CVB that are endemic in the U.S., and 10-30 percent have been infected with two or three serotypes (Pallansch, 1988). Infection results in a wide range of documentable illness only 10 percent of the time, but actual morbidity is likely much higher because many illnesses are not reported, do not require hospitalization, or the disease may have long latent periods, e.g., IDDM (Pallansch, 1988). Sadly, CVB infections in infants and young children can be fatal (Cherry, 1995).

Strains of several CVB serotypes can induce pancreatitis in mice, an excellent animal model of the disease in humans (Pappenheimer et al., 1951; Ross et al., 1974; Lansdown, 1976; Webb and Madge, 1980; Kuno et al., 1984). Severity of disease induced depends upon virulence determinants in the genotype of the infecting strain (Ramsingh et al., 1989; Caggana et al., 1993; Ramsingh and Collins, 1995). Molecular studies of one well-characterized strain of coxsackievirus B4 (CVB4) showed that changes in single amino acids in either of two of the four capsid polypeptides of the virion could significantly affect the capacity of the virus for inducing pancreatitis in mice (Caggana et al., 1993; Ramsingh and Collins, 1995). Infection of mice by a CVB4 can profoundly alter lymphocyte repertoires prior to onset of either hypoglycemia or hyperglycemia (Chatterjee et al., 1992) and autoantibodies are induced that cross react with viral antigens and glutamic acid dehydrogenase (GAD₆₅) in beta cells of the islets of Langerhans (Atkinson et al., 1994; Hou et al., 1994; Jones and Crosby, 1996; Lonnrot et al., 1996). Increased expression of GAD₆₅ in the islets of CVB4-infected mice has been found during active early virus infection at that site. The genetic background of the murine strain involved in the CVB4-mouse response to infection also plays a major role in determining whether or not pancreatitis/IDDM is induced and severity of disease induced (Lansdown, 1976; Webb and Madge, 1980; Ramsingh et al., 1989; Tisch et al., 1993).

* To whom all correspondence should be addressed.

Pancreatitis describes a diverse group of diseases in which injury to acinar cells of the exocrine pancreas precedes an inflammatory response directed toward the pancreas (Grendell and Egan, 1987; Steer and Meldolesi, 1988; Regan and Go, 1990; Leach et al., 1992; Bockman, 1997). Although the etiology of pancreatitis is most likely multiple, the majority of diagnosed cases of pancreatitis have been associated with either biliary obstruction or chronic alcohol abuse (Reber, 1987; Bilchik et al., 1990; Regan and Go, 1990). Over one-third of the 80,000 annual cases in the U.S. are idiopathic, or attributed to several clinical situations, including infections (Reber, 1987; Bilchik et al., 1990; Regan and Go, 1990). Chronic alcohol abuse is considered a major co-factor, as up to 74 percent of patients are considered alcoholics (Reber, 1987). Current clinical treatment for pancreatitis has not changed over the past twenty years and largely consists of nonspecific supportive therapy (Grendell and Egan, 1987; Leach et al., 1992).

The human pancreas has evolved into an organ with remarkable regenerative capabilities following acute pancreatitis (Bockman, 1997). Acinar cells of the exocrine pancreas can undergo dedifferentiation to form ductules and tubular complexes (Hall and Lemoine, 1992; Bockman, 1997). Epithelial cells in the ducts and tubular complexes apparently have pleuripotential capabilities (Lechene et al., 1991; Iovanna et al., 1992), and can differentiate to become several types of cells comprising the pancreas, including endocrine cells (Gu et al., 1994). Thus in the regenerating pancreas, acinar cells have multifunctional capabilities that are highly significant to the recovery process (Bockman, 1995; Bockman, 1997).

In a retrospective study of the effects of nutraceutical dietary intervention in humans with IDDM or noninsulin-dependent diabetes mellitus, a mixture of glyconutritional (Ambrotose®), phytonutritional (Phyt-Aloe™), phyto-genin (PLUS) and food-based vitamins/minerals was found to have significant benefits for these patients (McDaniel et al., 1997). The benefits included increases in health and an improved sense of well-being, decreases in Candida infections, decreases in other recurrent/persistent infections, resolution of vision problems, reduction in high blood pressure, and resolution of gastrointestinal tract problems and numbness, pain or burning in the lower extremities (McDaniel et al., 1997). The challenge of understanding the mechanism(s) by which supplemental dietary nutraceuticals are beneficial in recovery from acute pancreatitis suggested an examination of the potential of parenteral administration of the glyconutrient mixture Ambrotose® on coxsackievirus B3 (CVB3)-induced long-term (8 months) pancreatitis in a murine model of the disease. Data from the present study suggest that Ambrotose® did have an ameliorative effect in this virus-animal model, perhaps as an antioxidant working through macrophages. Whereas free glutathione (GSH) levels in splenic monocytes/macrophages were generally depressed in CVB3-inoculated mice, compared to normal mice, over an 8 month period of study, weekly inoculations of Ambrotose® restored free glu-

tathione levels in these cells to levels found in macrophages/monocytes from normal mouse spleens.

MATERIALS AND METHODS

Mice. Male adolescent (4 weeks of age) mice of the CD-1 strain were purchased from Charles River Breeding Laboratories, Boston, MA. All mice were maintained in Laboratory Animal Resources facilities of the University of Texas Health Science Center at San Antonio. Water and laboratory mouse chow were available *ad libitum* to all mice. Mice were rested three days after arrival.

Virus. The highly myocarditic CVB₃ variant is a Nancy strain prototype (Trousdale et al., 1979). CVB₃ was propagated and assayed for infectious virus titers by a plaque method in HeLa cells (Gauntt et al., 1995).

Ambrotose®. Bulk Ambrotose® was provided as a lyophilized powder, filler-free, Lot #550-1080, from Mannatech, Inc., Coppel, TX and rehydrated with sterile DPBS on the day of use.

Histology. Coronal sections of pancreata were stained with hematoxylin and eosin and duplicate sections scored blind by two reviewers for extensive ($\geq 90\%$) acinar cell destruction or categories of $<90-50$, $50-10$ or no scores given on acinar cell destruction at magnifications of 100 and 200X. Sections of pancreata reflect a conservative consensus of outcome; where there was a disagreement on % cells destroyed, the lower number was reported.

Virus neutralization assays. The highly sensitive cytopathic effects (cpe)-inhibition assay for anti-CVB3 antibodies was previously described (Godeny et al., 1988). In this assay, a volume of virus at 2×10^5 pfu/ml was mixed with an equal volume of a dilution of a serum (heat-inactivated) and the sealed mixture was held in a 37° incubator for 30 min. All dilutions of sera were made using virus growth medium (VGM; MEM containing 1% heat-inactivated fetal bovine serum and antibiotics). Aliquots (0.1 ml) of each mixture were inoculated in triplicate onto monolayer cultures of 10-20,000 HeLa cells per well in 96 well tissue culture plates, and the cultures were incubated for 24-36 hr at 37° until the virus control culture exhibited complete destruction. Cultures receiving diluted serum-virus mixtures were then scored as (-) for complete protection, 1+ to 3+ for partial protection of cells or (4+) which indicated no protection of cells, i.e., total cell destruction. The serum dilution which protected at least 50% or greater (2+ score) of cells in at least 2 of the 3 wells was taken as endpoint, and the reciprocal of the mean of the dilution at endpoint was taken as the titer.

Glucose concentrations. Glucose concentrations were determined in triplicate on heat-inactivated serum samples using an Ames Glucometer 3™ (Miles Inc., Diagnostics Division Elkhart, IN) according to the manufacturers directions. Values obtained by this method were very similar ($\leq 5\%$) to values obtained by a standard hexokinase method (Bondar and Mead, 1974).

Cellular glutathione levels. Cellular glutathione (GSH) levels were evaluated in attached splenic monocytes/macrophages with monochlorobimane (mBCI; Molecular Probes, Inc., Eugene, OR) using a laser scanning Meridian Ultima confocal microscope (Meridian Instruments, Inc., Okemos, MI). The cell-permeant mBCI probe is non-fluorescent, but forms a fluorescent conjugate with GSH in a reaction catalyzed by glutathione S-transferase (Rice et al., 1986; Shrieve et al., 1988). The microscope was used at an excitation wavelength of 351-363 nm, and emitted fluorescence at 461 nm was detected using a barrier filter (BP 485/45). Excitation and detection parameters were kept constant for all GSH experiments. For image capture, the laser excited fluorescence in cells from a two-dimensional raster pattern (scan parameters were optimized for maximum detection of fluorescence with minimum cellular photobleaching). Kinetic analysis of mBCI-GSH conjugation was performed at room temperature on the Ultima stage by recording changes in fluorescence intensity at 15-sec intervals for approximately 15 min (Barhoumi et al., 1995; Busbee et al., 1999).

Experimental protocol. After resting for 3 days after arrival, mice were injected with Ambrotose® at 10mg/kg body weight, diluted in Dulbecco's phosphate-buffered saline (DPBS), or DPBS alone on day -7 by the intraperitoneal (i.p.) route. On day 0, half of the group of mice administered Ambrotose® and half of the control (DPBS only) groups were inoculated with 5×10^5 plaque-forming units (PFU) of virus by i.p. route. The remaining half of each group were administered DPBS only. Approximately every 7 days thereafter, half of all mice were injected by i.p. route with Ambrotose® at doses of 10mg/kg body weight or the other half were injected with DPBS. On select days post-inoculation (p.i.), each mouse was weighed. Generally seven randomly selected mice were deeply anesthetized (Metafane) and humanely sacrificed. Sera from individual mice were stored for antiviral antibody titer assays. Pancreata were quickly removed and fixed in buffered 10% formalin. Spleens were removed and processed to obtain attached monocytes/macrophages for GSH levels as above.

RESULTS

Effect of Parenteral Administration of Ambrotose® on Total Body Weights in Normal and Virus-Challenged Mice. Over the first five weeks post-inoculation of virus, most virus-challenged mice exhibited a significant total body weight loss, compared to normal mice (Table 1). Thereafter, virus-challenged mice had similar total body weights as normal mice. Beginning around week four post-virus challenge and for the next three weeks, the group of mice receiving Ambrotose® showed significant recovery in whole body weight losses to achieve body weights similar to those found in normal mice; this weight recovery remained for the next 6 months. However, normal mice receiving Ambrotose® also showed significant weight gain beginning around week four and this significantly increased

weight (greater than that of normal mice) was maintained for the next six weeks. Thereafter, weight differences were not always significant between the two groups. The transient six week weight gain promoted by Ambrotose® in normal mice amounted to about an 8% increase in total body weight.

Ambrotose® Effects Faster Recovery from CVB3-Induced Pancreatitis in Mice. Islets showing inflammation were found rarely and sporadically in pancreata of CVB3_m-inoculated mice, regardless of whether the mice were exposed to Ambrotose® or not (Gauntt and Wood, data not shown). Weekly administration of Ambrotose® generally appeared to reduce both the number of pancreas sections with any acinar cell destruction and the severity of acinar cell destruction per pancreas section, compared to similar parameters in the virus-challenged control group (Table 2). Photomicrographs of typical stained (hematoxylin and eosin) sections of pancreas that show five categories of % of acinar cells destroyed in pancreata of CVB3-inoculated mice ($\geq 90\%$; $<90-50\%$; $<50-10\%$; $<10\%$; and none) are presented in Figure 1. On day 28, four weeks after virus challenge there were no significant differences in either parameter between Ambrotose®-treated and nontreated groups of CVB3_m-inoculated mice. It was not until week 7 post virus challenge that the proportion of acinar cells (two sections per mouse pancreas) in mice given Ambrotose® showed a significant reduction in pathologic alterations. Thereafter, pancreata taken from Ambrotose®-treated groups of mice at three of four times of sacrifice had either a significant reduction in number of sections of pancreata with any destruction of acinar cells (weeks 10, 19 and 35.5 post-inoculation) or both a reduction in number of pancreas sections with any acinar cell destruction and a reduction in proportion of acinar cells destroyed to $<10\%$ or none (week 19), compared to groups of virus-challenged mice not receiving injections of Ambrotose®. Note that at 5 of 6 times of assay (day 70 being the exception), levels of GSH in splenocytes from virus-infected mice were reduced to 67- 79% of levels found in splenocytes from normal mice. In contrast, note that at the same 5 times of assay, splenocytes from Ambrotose®-treated virus-challenged mice contained 19-97% more GSH than splenocytes from untreated virus-challenged (virus control) mice. These data also show that there were a few mice in each of the groups receiving Ambrotose® treatment that derived no benefit from the glyconutritional mixture, regardless of the number of weeks of dietary supplementation. One mouse even had extensive destruction of pancreatic acinar cells after Ambrotose® administration continuously through week 35.5. Because no mice died during the experiment and Chi Square comparative analyses of the effect of Ambrotose® on virus-induced destructive processes relative to the proportion of pancreas sections with no or $<10\%$ of acinar cells destroyed versus the extent of pathology in the pancreata of control mice showed significant differences ($p=0.05$), the data suggest that some outbred mice, like some humans (McDaniel et al., 1997) cannot derive health recovery

Table 1. Total Body Weight in Male CD-1 Mice Inoculated with Ambrotose® ± Coxsackievirus B3 (CVB3)*

Day post inoculation virus	Body Weights Under Several Conditions for Groups of Mice (Mean weight(g)± S.D.)			
	Normal	Normal+ Ambrotose ⁷	Virus	Virus+ Ambrotose ⁷
-7		25.4 ± 1.2		
0	27.6 ± 0.8	28.0 ± 0.7	27.6 ± 1.1	27.9 ± 1.1
7	29.8 ± 1.7	30.8 ± 2.1 ^a	27.6 ± 1.9 ^e	28.4 ± 1.5 ⁱ
14	31.9 ± 2.1	32.8 ± 2.3	29.3 ± 3.7 ^f	30.5 ± 2.7 ^k
21	33.8 ± 2.3	34.9 ± 2.4	31.7 ± 4.0 ^f	32.9 ± 3.5 ^k
28	34.6 ± 2.5	36.3 ± 2.6 ^b	32.8 ± 4.0 ^{e,i}	34.7 ± 3.3 ⁿ
35	35.3 ± 2.6	37.8 ± 2.8 ^c	34.0 ± 4.4 ^{f,p}	35.6 ± 1.6 ^l
42	35.6 ± 2.1	37.9 ± 2.9 ^b	34.3 ± 4.2 ^l	36.3 ± 3.3
49	37.7 ± 2.8	39.7 ± 2.9	37.7 ± 4.9	38.2 ± 3.2
56	37.5 ± 2.8	40.3 ± 3.3 ^c	37.0 ± 4.1	38.0 ± 3.8 ⁿ
63	38.6 ± 3.1	41.4 ± 3.8 ^c	38.2 ± 4.2 ^m	40.1 ± 4.0
70	39.5 ± 3.7	42.7 ± 3.9 ^c	39.0 ± 5.1	40.3 ± 4.2
77	39.9 ± 3.9	42.5 ± 4.2	40.6 ± 5.5	41.7 ± 3.8
84	40.6 ± 4.1	43.4 ± 4.1	40.8 ± 5.0	42.0 ± 4.1
92	41.4 ± 4.5	44.4 ± 4.9	40.2 ± 5.6 ⁿ	43.5 ± 4.6
99	42.4 ± 5.0	45.8 ± 4.8 ^b	43.1 ± 5.7	45.0 ± 4.6
105	42.1 ± 5.0	44.7 ± 4.7	43.3 ± 5.9	44.4 ± 4.6
112	42.3 ± 4.0	46.0 ± 4.3 ^a	46.4 ± 4.5 ^{e,i}	43.0 ± 5.4
120	43.0 ± 4.3	46.5 ± 4.4	47.3 ± 4.4	46.5 ± 5.6 ^q
125	43.6 ± 3.8	46.6 ± 4.5	46.5 ± 4.1 ^d	46.6 ± 5.4
133	43.9 ± 4.3	47.8 ± 4.4 ^b	47.2 ± 4.5	46.5 ± 5.4

* On day -7, 25 random mice were weighed before assignment to one of the four groups. Half of the mice were injected with buffer (DPBS) and half with Ambrotose® at 10mg/kg body weight in DPBS on day -7 post-inoculation (p.i.). On day 0, one group of normal mice (Virus group) and one group of Ambrotose® injected mice were challenged with 5x10⁵ plaque-forming units of purified CVB3 per mouse. On designated days p.i., all mice in each of the groups were weighed and the number decreased as 7 mice per group were withdrawn for sacrifice. Thus, up through day 28 p.i., there were 45 mice/group; through day 49 p.i., 38 mice per group; through day 70 p.i., 31 mice/group; through day 105 p.i., 24 mice per group and through day 133 p.i., 17 mice/group. After weighing, mice were inoculated with DPBS or Ambrotose® on the day indicated.

^{a-p}Statistical analyses for significant differences between two groups:

normal vs normal + Ambrotose® at p<0.05^a, p<0.02^b,

p<0.01^c

normal vs virus at p<0.05^d, p<0.02^e, p<0.01^f

normal vs virus + Ambrotose® at p<0.05^g

normal + Ambrotose® vs virus + Ambrotose® at

p<0.05^h, p<0.02ⁱ, p<0.01^j, p<0.001^k

virus vs virus + Ambrotose® at p<0.05^l,

p<0.02^m, p<0.01ⁿ, p<0.001^p

benefits in some diseases via supplementation of the diet with the glyconutritional Ambrotose®.

Serum Glucose Levels. CVB3_m challenge of male CD-1 mice did not affect levels of glucose in these mice between four and 35.5 weeks post-inoculation (Table 3). These data confirm previous findings (Reyna, Wood and Gauntt, unpublished data) that CVB3_m challenge of this strain (CD-1) of mouse does not induce diabetes mellitus type I. Administration of Ambrotose® weekly to normal or virus-inoculated mice also did not significantly alter glucose levels.

Effect of Ambrotose® Injections on Anti-CVB3 Antibody Titers. Recent work (Gauntt et al., 1999) has shown that aloe polymannose significantly enhanced anti-CVB3_m antibody titers in mice and previous studies showed that mice that produced faster or higher titers of anti-CVB3 antibodies in response to virus challenge had reduced virus-induced acute cardiopathologic alterations com-

pared to mice that delayed or had reduced production of anti-CVB3 antibodies (Gauntt, 1997). Therefore, it was of interest to assess antiviral antibody titers in mice given Ambrotose®, particularly to determine if this glyconutritional mixture could stimulate anti-CVB3 antibody titers at early times post virus-challenge. The data (Table 4) show that serum CVB3-neutralizing antibody titers from individual mice varied over a wide range at each time of assay, leading to very large standard deviations for the small geometric mean antibody titers. Ambrotose® administration had no effect on anti-CVB3 antibody titers at any time over this 8 month experiment.

Ambrotose® Injections Increased the Level of Free Glutathione [GSH] in Splenic Macrophages. Many viruses (Reiss and Komatsu, 1998), including CVB3 (Freeman et al., 1998), induce synthesis of nitric oxide synthetase during infection of cells. CVB3-infected cells in murine heart tissues infected in vivo also attract macrophages

Table 2. Effect of Ambrotose® on Coxsackievirus B3-Induction of Pathology in Pancreata of CD-1 Mice*

Day p.i.	Treatment with Ambrotose [†]	Number of sections examined	Histopathology of the pancreas, percentage of sections presenting with extent of acinar cell destruction (% destroyed)					Percentage of sections with: any extent of acinar cell destruction	Percentage of sections with: destruction of <10% of cells or none	Average % of acinar cells destroyed in all sections per group (mean ± S.E.M.) [‡]
			(>90)	(<90-50)	(50-10)	(<10)	(none)			
28	NO	14	0	0	50	29	21	79	50	29.4 ± 6.5
	YES	14	0	7	29	50	14	86	64	12.6 ± 4.2 ^a
49	NO	14	14	14	29	14	29	71	43	66.3 ± 14.0
	YES	14	0	0	0	71	29	71	100 ^{†0.001}	2.6 ± 0.6 ^c
70	NO	14	7	0	64	29	0	100 ^{†0.001}	9	26.9 ± 6.0
	YES	14	29	14	0	0	57	43	7	39.6 ± 12.4
105	NO	14	21	0	21	29	21	77	54	27.2 ± 11.3
	YES	14	14	0	14	43	29	71	71	19.1 ± 9.1
133	NO	14	0	21	43	21	14	86	36	25.3 ± 6.0
	YES	14	0	0	14	36	50	50 ^{0.009}	86 ^{0.008}	4.2 ± 1.8 ^b
248	NO	16	0	6	44	44	6	94 ^{0.008}	50	16.6 ± 4.8
	YES	19	5	5	16	26	47	53	74	13.4 ± 5.4

* Mice were injected with 10mg/kg of Ambrotose® in DPBS or DPBS only by intraperitoneal route 7 days prior to challenge with CVB3_m on day 0. On day 0 and approximately every 7 days thereafter, mice were inoculated with Ambrotose® or DPBS through day 241 p.i. Two coded sections, 40-60 microns apart, of each pancreas were examined for acinar cell destruction by two observers at 40 and 100X magnification. The data presented represent a conservative consensus of the outcomes, i.e., where there was disagreement on the % cells destroyed, the lower number was used.

† Fisher's exact test; p = a superscript number indicating a significant difference between groups of mice given Ambrotose® and those not receiving Ambrotose® in the virus control.

‡ Student's t test comparative analysis of average percentages of acinar cells destroyed in all sections in the virus control group vs the group of virus-challenged mice injected with Ambrotose® as a dietary supplement: p<0.05^a, p<0.01^b or p<0.001^c

and neutrophils that express high levels of this enzyme (Hiraoka et al., 1996; Lowenstein et al., 1996), thus increasing the overall levels of NO, an oxidant (Dawson and Dawson, 1994) in the infected tissue. Dietary supplementation with a variety of antioxidant materials has proven beneficial in a variety of diseases (Maxwell, 1995; Barhoumi et al., 1997). Cell systems designed to oppose toxic effects of oxidative radicals include superoxide dismutase, catalase, glutathione (GSH) and glutathione peroxidase. The following experiments determined whether CVB3-challenged mice given Ambrotose® by parenteral injections had enhanced GSH levels in their splenic monocytes/macrophages, compared to the CVB3-challenged virus controls, as one possible explanation for how Ambrotose® contributed to recovery from pancreatitis.

Data on free GSH levels in splenic monocytes/macrophages at six time points from all four groups of mice are presented in Table 5. Ambrotose® administration to normal mice did not alter GSH levels. Splenic macrophages/monocytes from mice previously challenged with CVB3_m generally, i.e., five of six sample times, had lower GSH levels than those found in the normal group of mice at each time of sacrifice. Virus-infected mice administered Ambrotose® contained splenic macrophages/monocytes with significantly enhanced GSH levels in the same five of six sample times cited above. The increased GSH levels in splenic macrophages/monocytes taken from the virus-challenged mice given Ambrotose® ranged from about 9 to 97% above the

levels found in virus-control mice. We conclude that Ambrotose® administration to CVB3_m-inoculated mice significantly increased GSH levels in their splenocytes.

DISCUSSION

The data show that inoculation of mice with a CVB3 strain can result in long term pancreatitis in some mice whereas other mice appear to resolve the acute pancreatitis, i.e., the destruction of acinar cells occurred over a range of >90% destroyed to none destroyed in groups of mice sacrificed at times up to 8 months post-CVB3 inoculation. No mice died during the course of this experiment and some presented with near total pancreatic acinar cell destruction at their time of sacrifice, including one mouse at eight months post virus-challenge. The latter mouse was from the group that received weekly i.p. Ambrotose® injections during the 8 month experiment, suggesting that this mouse, and some other mice in the Ambrotose®-injected groups, were incapable of deriving any ameliorative benefit for their pancreata from the glyconutritional mixture. It is not known why some mice, like some humans (30), do not derive health benefits from dietary nutritional supplements. In contrast, several types of statistical analyses (Chi square, Tukey's Multiple Comparison or Student's t test) of the data on all mice or on specific time-of-sacrifice groups of CVB3-challenged Ambrotose®-injected versus control groups showed there was a significant reduction in acinar cell destruction and increased glutathione levels in splenic macrophages in glyconutrient

Figure 1a

Figure 1b

Figure 1c

Figure 1d

Figure 1e

Figure 1f

Figure 1. Photomicrographs of typical fields of pancreata displaying varying degrees of CVB₃-induced destruction of acinar cells. These representative fields show the range of acinar cell destruction (% cells destroyed) in the pancreas of individual virus-challenged mice $\pm$ Ambrotose[®]. Information for each panel includes: an assessment of the range of % of cells destroyed, whether Ambrotose[®] was injected (+) or not (-), and the day of sacrifice p.i.: a) >90% cells destroyed, (+), day 28; b) <90 to 50% cells destroyed, (-), day 133; c) <50 to 10% cells destroyed, (+), day 133; d) <10% cells destroyed, (+), day 248; e) no cell destruction, (+), day 105; and (f) note the severe destruction of one area of tissue (bottom of field) and no destruction in the adjacent tissue, (+), day 248. The photomicrograph shown in e) is indistinguishable from photomicrographs of pancreata taken from normal mice not exposed to virus. Magnification is 551 X.

Table 3. Serum Glucose Levels in CVB3_m-Inoculated CD-1 Mice ± Treatment with Ambrotose®

Day post-CVB3 _m challenge	Age of mice (weeks)	Mean serum glucose levels (mg/dl ± S.D.) for (n) mice per group*			
		normal (control)	normal + Ambrotose [†]	virus-inoculated	virus-inoc. + Ambrotose [‡]
28	8	†<269 ± 23(6)	<314 ± 38(6)	<270 ± 72(3)	<270 ± 52(6)
49	15	251 ± 22(7)	266 ± 28(7)	<235 ± 23(6)	259 ± 23(7)
70	18	243 ± 54(7)	263 ± 41(7)	217 ± 27(6)	264 ± 49(7)
105	23	122 ± 19(7)	123 ± 25(6)	185 ± 57(5)	175 ± 40(7)
133	27	247 ± 59(7)	215 ± 43(7)	<248 ± 57(6)	<226 ± 28(6)
248	39	<150 ± 17(7)	161 ± 23(9)	<161 ± 40(7)	199 ± 35(10)

* Half of all mice were injected i.p. with Ambrotose® (10mg/kg body weight) dissolved in DPBS and the remaining half received DPBS beginning on day -7 and continuing approximately every 7 days through day 241 p.i.; inoculations were in 0.2 ml. On day 0, half of each of the latter two groups were challenged with CVB3_m at 5x10⁵ pfu/mouse in 0.2 ml by the i.p. route. Seven mice from each group were sacrificed on a given day, blood was processed to obtain sera for assay of glucose.

† Glucose concentrations were assessed by an Ames Glucometer 3.

‡ indicates that one or more samples within the group contained <15mg/dl glucose.

Table 4. Effect of Ambrotose® Supplementation to the Diet on Anti-Coxsackievirus B3 (CVB3) Neutralizing Antibody Titers in CD-1 Mice Inoculated with CVB3*

Condition	Day post-inoculation	Individual mouse titers (geometric mean)	Group Mean (± S.D.)
Virus + Ambrotose®	28	101, 40, 127, 101, 9, 101, 160	91 ± 51
	49	64, 80, 32, 40, 101, 127, 4	64 ± 42
	70	20, 32, 254, 101, 320, 9, 80	117 ± 122
	105	9, 101, 160, 201, 403, 40, 80	142 ± 133
	133	160, 201, 50, 640, 80, 32, 9	167 ± 220
	248	40, 320, 50, 805, 160, 160, 25, 9, 20, 40	181 ± 243
Virus	28	32, 25, 4, 127, 2, 320, 25	76 ± 115
	49	25, 101, 64, 50, 127, 50, 25	63 ± 38
	70	25, 2, 50, 25, 80, 25, 80	41 ± 30
	105	160, 64, 201, 201, 2, 201, 80	130 ± 81
	133	40, 101, 80, 25, 403, 80, 20	107 ± 134
	248	160, 80, 50, 50, 80, 101, 403, 805	216 ± 265

* Mice were injected i.p. with Ambrotose® at 10mg/kg body weight every 7-8 days beginning on day -7. On day 0 all mice were inoculated i.p. with 5x10⁵ pfu of CVB3_m. Mice were sacrificed at the indicated times. Sera was heat-inactivated (56°, 30 min) prior to assay for anti-CVB3 neutralizing antibody titers by a cytopathic effects-inhibition microtiter assay in triplicate using HeLa cells. Values shown represent geometric means; there were no titer discrepancies among the three cultures greater than a one dilution difference. For calculation purposes, a single culture with a reading of <20 was arbitrarily assigned a value of 2.

Table 5. Effect of Weekly Ambrotose® Administration on Free Glutathione [GSH] Levels in Splenic Monocytes/ Macrophages from Coxsackievirus B3-Inoculated CD-1 Mice*

Day post-inoculation of virus	Mean units (± S.D.) of free GSH per group:					
	normal	normal + Ambrotose®	virus-infected	% of normal control values	virus-infected + Ambrotose®	% of virus-infected control values
28	26.0 ± 5.7 (7) [†]	19.8 ± 6.8 (7)	17.4 ± 7.6 (7)	67	34.2 ± 9.6 (7)	197
49	1181 ± 536 (108)	1184 ± 579 (144)	794 ± 380 (101)	67	1527 ± 688 [‡] (114)	192
70	1364 ± 523 (26)	1344 ± 409 (250)	1314 ± 217 (458)	96	1431 ± 548 (310)	109
105	1606 ± 271 (216)	1475 ± 342 (217)	1270 ± 512 (181)	79	1506 ± 321 [‡] (242)	119
133	1116 ± 300 (179)	1115 ± 295 (221)	875 ± 280 (156)	78	1194 ± 300 [‡] (204)	136
248	1251 ± 572 (117)	1428 ± 643 (101)	835.5 ± 435 (118)	67	1621.5 ± 565 [‡] (117)	194

* Adolescent male CD-1 mice were inoculated by i.p. route with 5X10⁵ plaque-forming units of purified CVB3_m on day 0. Mice were injected i.p. with 10mg/kg body weight of Ambrotose® (A) on days -7, 0 and approximately every 7 days thereafter through day 241 p.i. Relative free GSH levels, fluorescence units as a function of mBCL binding, were measured 5-20 times per each of the splenic cell samples from 7 individual mice per sample through day 133 p.i.; on day 248 p.i. there were 8, 9, 8 and 10 mice in the N, N + A, VC and V + A groups, respectively. Low mean units reported for samples taken on day 28 were due to a technical problem of cell plating which was consistent in all four samples to yield reliable data.

† Numbers in parenthesis represent the independent number of GSH measurements made in random cells obtained from that group of mice.

‡ Values are significantly different from the values of the virus control group (p<0.05) on that day of assay by Tukey's Multiple Comparison Test.

treated mice. Thus, these data lead to the conclusion that some mice benefited in their recovery from pancreatitis due to their treatment with Ambrotose®.

Data presented herein suggest one potential recovery mechanism that may be promoted by Ambrotose®, via an unknown mechanism, is to increase levels of glutathione, a major antioxidant in cells. Previous studies (Barhoumi et al., 1997, Busbee et al., 1999) showed that Ambrotose® provided protection against chemical depletion of glutathione in rat liver cells by patulin, a sulfhydryl-binding reagent, via an unknown blocking mechanism. Competitive binding of patulin by a glyconutrient(s) in the mixture comprising Ambrotose®, is one possible explanation.

Blocking glutathione depletion could significantly contribute to prevention of the oxidant activity from nitric oxide (NO) induced by CVB3 replication in cells or from inflammatory cells attracted to the site of replication of the virus in an animal, e.g., the heart or pancreas (Huber et al., 1998). Inhibitors of nitric oxide synthetase lead to increased titers of virus in the heart and higher mortality in CVB3-infected mice (Hiraoka et al., 1996; Lowenstein et al., 1996), thus NO must play an unknown role in controlling the extent of virus replication in tissues in vivo. However, NO can react with oxygen to result in free radical formation and can participate in tissue injury. Mice challenged with encephalomyocarditis virus, a naturally-occurring murine picornavirus, can develop diabetes mellitus type 1, but treatment of these mice with an inhibitor of inducible NO synthetase reduced the incidence of diabetes (Hirasawa et al., 1997). Thus, i.p. injection of Ambrotose® to increase, maintain, or restore intracellular levels of antioxidants such as glutathione would provide time for regeneration of acinar cells to replace those destroyed by virus-induced direct (replicative) and indirect (inflammatory) mechanisms.

The benefits of adding nutraceuticals to the diets of some patients with either types of diabetes have been previously described (McDaniel et al., 1997). Some parameters of that study showed significant improvements with the dietary supplement whereas others showed minimal improvement similar to the outcome for mice described in the present study. The complexity of health parameters in individuals (mice or humans) would dictate that a broad range of outcomes is likely, as was found. Further work is needed to define why some individuals respond more favorable to glyconutritional supplementation than others.

ACKNOWLEDGEMENTS

We thank Marguerite Starr for her excellent and patient assistance in the preparation of this manuscript. These studies were funded by grants from the Fisher Institute for Medical Research, Grand Prairie, TX and from the ERACE Foundation of Los Angeles, CA.

REFERENCES

- Atkinson, MA, Bowman, MA, Campbell, L, Darrow, BL, Kaufman, DL, and Maclaren, NK: Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes. *J. Clin. Invest.*, 94:2125-2129, 1994.
- Barhoumi, R, Bailey, RH, and Burghardt, RC: Kinetic analysis of glutathione in anchored cells with monochlorobimane. *Cytometry*, 19:226-234, 1995.
- Barhoumi, R, Burghardt, RC, Busbee, DL, and McDaniel, HR: Enhancement of glutathione levels and protection from chemically initiated glutathione depletion in rat liver cells by glyconutritional. *Proc. Fisher Inst. Med. Res.*, 1:12-16, 1997.
- Bilchik, AJ, Leach, SD, Zucker, KA, and Modlin, IM: Experimental models of acute pancreatitis. *J. Surg. Res.*, 48:639-647, 1990.
- Bockman, DE: Toward understanding pancreatic disease: from architecture to cell signaling. *Pancreas*, 11:324-329, 1995.
- Bockman, DE: Morphology of the exocrine pancreas related to pancreatitis. *Microsc. Res. Tech.*, 37:509-519, 1997.
- Bondar, RJL, and Mead, DC: Evaluation of glucose-6-phosphate dehydrogenase from *Leukonostoc mesenteroides* in the hexokinase method for determining glucose in serum. *Clin. Chem.*, 20:586-592, 1974.
- Busbee, D, Gauntt, C, Mouneimne, R, Burghardt, RC, McAnalley, and McDaniel, HR: Protection from glutathione depletion by a glyconutritional mixture of saccharides, *J. Am. Aging Assoc.* (Pending), 1999.
- Caggana, M, Chan, P, and Ramsingh, A: Identification of a single amino acid residue in the capsid protein VP1 of coxsackievirus B4 that determines the virulent phenotype. *J. Virol.*, 67:4797-4803, 1993.
- Chatterjee, NK, Hou, J, Dockstader, P, and Charbonneau, T: Coxsackievirus B4 infection alters thymic, splenic, and peripheral lymphocyte repertoire preceding onset of hyperglycemia in mice. *J. Med. Virol.*, 38:124-131, 1992.
- Cherry, JD: Enteroviruses, in *Infectious Diseases of the Fetus and Newborn Infant*, edited by Remington, JS, Klein, JD, 4th Ed., Philadelphia, W.B. Saunders, 1995, pp. 404-446.
- Dawson, TM, and Dawson, VL: Nitric oxide: actions and pathologic roles. *Neuroscientist*, 1:9-20, 1994.
- Freeman, G, Colston, J, Zabalgoitia, M, and Chandrasekar, B: Contractile depression and expression of proinflammatory cytokines and iNOS in viral myocarditis. *Am. J. Physiol.*, 274:H249-H258, 1998.

- Gauntt, CJ: Roles of the humoral response in CVB-induced disease, in *The Coxsackie B Viruses*, Volume 223, Current Topics in Microbiology and Immunology, edited by Tracy, S, Chapman, N and Mahy, B, Berlin, Springer, 1997, pp. 259-282.
- Gauntt, CJ, Arizpe, HM, Higdon, AL, Wood, HJ, Bowers, DF, Rozek, MM, and Crawley, R: Molecular mimicry, anti-coxsackievirus B3 neutralizing monoclonal antibodies and myocarditis. *J. Immunol.*, 154:2983-2995, 1995.
- Gauntt, CJ, Wood, HJ, McDaniel, HR, and McAnalley, BH: Aloe polymannose enhances anti-coxsackievirus antibody titers in mice. *Phytotherapy Res.*, 14:1-6, 2000.
- Godeny, EK, Arizpe, HM, and Gauntt, CJ: Characterization of the antibody response in vaccinated mice protected against coxsackievirus B3-induced myocarditis. *Viral Immunol.*, 1:305-314, 1988.
- Grendell, JH, and Egan, J: Acute pancreatitis. *West J. Med.*, 146:598-602, 1987.
- Gu, D, Lee, M-S, Krah, T, and Sarvetnick, N: Transitional cells in the regenerating pancreas. *Development*, 120:1873-1881, 1994.
- Hall, PA, and Lemoine, NR: Rapid acinar to ductal transdifferentiation in cultured human exocrine pancreas. *J. Pathol.*, 166:97-103, 1992.
- Hiraoka, Y, Kishimoto, C, Takada, H, Nakamura, M, Kurokawa, M, Ochiai, H, and Shiraki, K: Nitric oxide and murine coxsackievirus B3 myocarditis: aggravation of myocarditis by inhibition of nitric oxide synthetase. *J. Am. Coll. Cardiol.*, 28:1610-1615, 1996.
- Hirasawa, K, Jun, H, Maeda, K, Kawaguchi, Y, Itagaki, S, Mikami, T, Baek, H, Doi, K, and Yoon, J: Possible role of macrophage-derived soluble mediators in the pathogenesis of encephalomyocarditis virus induced diabetes in mice. *J. Virol.*, 71:4024-4031, 1997.
- Hou, J, Said, C, Franchi, D, Dockstader, P, and Chatterjee, NK: Antibodies to glutamic acid decarboxylase and P2-C peptides in sera from coxsackie virus B4-infected mice and IDDM patients. *Diabetes*, 43:1260-1266, 1994.
- Huber, SA, Gauntt, CJ, and Sakkinen, P: Enteroviruses and myocarditis: viral pathogenesis through replication, cytokine induction and immunopathogenicity. *Advances Virus Res.*, 51:35-63, 1998.
- Iovanna, JL, Lechene de la Porte, P, and Dagorn, J-C: Expression of genes associated with dedifferentiation and cell proliferation during pancreatic regeneration and following acute pancreatitis. *Pancreas*, 7:712-718, 1992.
- Jones, DB, and Crosby, I: Proliferative lymphocyte responses to virus antigens homologous to GAD65 in IDDM. *Diabetologia*, 39:1318-1324, 1996.
- Kuno, S, Itagaki, A, Yamazaki, I, Katsumoto, T, and Kurimura, T: Pathogenicity of newly isolated coxsackievirus B4 for mouse pancreas. *Acta. Virol.*, 28:433-436, 1984.
- Lansdown, ABG: Pathological changes in the pancreas of mice following infection with coxsackie B virus. *Br. J. Exp. Pathol.*, 57:331-338, 1976.
- Leach, SV, Gorelick, FS, and Modlin, IM: New perspectives on acute pancreatitis. *Scand. J. Gastroenterol.*, 27:9-38, 1992.
- Lechene de la Porte, P, Iovanna, J, Odaira, C, Choux, R, Sarles, H, and Berger, Z: Involvement of tubular complexes in pancreatic regeneration after acute necrohemorrhagic pancreatitis. *Pancreas*, 6:298-306, 1991.
- Lonnrot, M, Hoyoty, H, Knip, M, Roivainen, M, Kulmala, P, Leinikki, P, and Akerblom, HK: Antibody cross-reactivity induced by the homologous regions in glutamic acid decarboxylase (GAD65) and 2C protein of coxsackievirus B4. *Childhood Diabetes in Finland Study Group. Clin. Exp. Immunol.*, 104:398-405, 1996.
- Lowenstein, C, Hill, S, Lafond-Walker, A, Wu, J, Allen, G, Landavere, M, Rose, N, and Herkowitz, A: Nitric oxide inhibits viral replication in murine myocarditis. *J. Clin. Invest.*, 97:1837-1843, 1996.
- Maxwell, SRJ: Prospects for the use of antioxidant therapies. *Drugs*, 49:345-361, 1995.
- McDaniel, CF, Dykman, KD, McDaniel, HR, Ford, CR, and Tone, CM: Effects of nutraceutical dietary intervention in diabetes mellitus: a retrospective survey. *Proc. Fisher Inst. Med. Res.*, 1:19-23, 1997.
- Melnick, JL: Polioviruses and other enteroviruses, in *Viral Infections of Humans. Epidemiology and Control*, edited by Evans, AS, and Kaslow, RA, 4th Ed., New York, Plenum Medical, 1997, pp. 583-663.
- Pallansch, MA: Epidemiology of group B coxsackievirus, in *Coxsackieviruses-A General Update*, edited by Bendinelli, M, and Friedman, H, New York, Plenum, 1988, pp. 399-417.
- Pappenheimer, AM, Kunz, LJ, and Richardson, S: Passage of coxsackie virus (Connecticut-5 strain) in adult mice with production of pancreatic disease. *J. Exp. Med.*, 94:45-64, 1951.
- Ramsingh, A, and Collins, DN: A point mutation in the Vp4 coding sequence of coxsackievirus B4 influences virulence. *J. Virol.*, 69:7278-7281, 1995.
- Ramsingh, A, Slack, J, Silkworth, J, and Hixon, A: Severity of disease induced by a pancreatropic coxsackie B4 virus correlates with the H-2K locus of the major histocompatibility complex. *Virus Res.*, 14:347-358, 1989.

Reber, HA: Alcoholic pancreatitis, in *Surgical Diseases of the Pancreas*, edited by Howard, JM, Jordan, GL, and Reber, HA, Philadelphia, Lea and Febiger, 1987, pp. 284-296.

Regan, PT, and Go, VLW: Pancreatic diseases, in *Internal Medicine*, edited by Stein, JH, 3rd Ed., Boston, Little, Brown and Co., 1990, pp. 546-556.

Reiss, CS, and Komatsu, T: Does nitric oxide play a critical role in viral infections. Minireview. *J. Virol.*, 72:4547-4551, 1998.

Rice, GC, Bump, EA, Shrieve, DC, Kovacs, W, and Kovacs, M: Quantitative analysis of cellular glutathione by flow cytometry using monochlorobimane: some applications to radiation and drug resistance in vitro and in vivo. *Cancer Res.*, 46:6105-6110, 1986.

Ross, ME, Hayashi, K, and Notkins, AL: Virus-induced pancreatic disease: alterations in concentration of glucose and amylase in blood. *J. Infect. Dis.*, 129:669-676, 1974.

Shrieve, DC, Bump, EA, and Rice, GC: Heterogeneity of cellular glutathione among cells derived from a murine fibrosarcoma or a human renal cell carcinoma detected by flow cytometric analysis. *J. Biol. Chem.*, 263:14107-14114, 1988.

Steer, ML, and Meldolesi, J: Pathogenesis of acute pancreatitis. *Ann. Rev. Med.*, 39:95-105, 1988.

Tisch, R, Yang, S-D, Singer, SM, Liblau, RS, Fugger, L, and McDevitt, HO: Immune response to glutamic acid decarboxylase correlates with insulinitis in non-obese diabetic mice. *Nature*, 366:72-75, 1993.

Trousdale, MD, Paque, RE, and Gauntt, CJ: Assessment of coxsackievirus B3 ts mutants for induction of myocarditis in a murine model. *Infect. Immun.*, 23:486-495, 1979.

Webb, SR, and Madge, GE: The role of host genetics in the pathogenesis of coxsackievirus infection in the pancreas of mice. *J. Inf. Dis.*, 141:47-54, 1980.