

Antiviral Effectiveness of Grape Juice

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ABSTRACT

Grape juice inactivated human enteroviruses, but not parainfluenza type 1 (Sendai) virus, *in vitro*. The effect was not one of aggregation or of degradation of the virus surface. Some of the inactivated virus adsorbed specifically to host cells, but did not infect them. Most of the inactivated virus could be reactivated by treatment with polyethylene glycol. Grape juice-inactivated virus and coproantibody-neutralized virus were both reactivated by contents of porcine stomach and duodenum, which suggests that ingestion of such viruses would lead to intestinal infection. Grape juice-inactivated virus was efficiently reactivated by human blood serum. Ingested grape juice has not been shown likely to prevent or modify human enterovirus infections.

In a series of reports, Konowalchuk and Speirs (5,6,7) described an *in vitro* activity against viruses that was common to several fruits and vegetables and their products. They called the phenomenon "inactivation" (and so shall we), although it differs from most known modes of virus inactivation in that the virus particle is apparently not degraded in the process. Much of their attention was devoted to grapes, grape juices, and wines. The prospect that these foods might prevent or cure virus infections in humans attracted a good deal of attention in the popular and scientific press. The coverage ranged from serious to clearly frivolous.

Given the hopes that might have been built on these reports, we thought it important to try to confirm the findings of Konowalchuk and Speirs and to assess the significance of these findings to human health. Our work was done almost exclusively with heat-pasteurized Concord grape juice. In its simplest hypothetical mode of action, grape juice might exert its protection locally in the digestive tract by inactivating virus before or after ingestion. Alternately, the active substances from the grape juice would have to pass into the bloodstream to prevent virus from being transported in the blood or to reach and protect remote sites of virus infection, such as the respiratory tract. We chose not to consider any means of administration of the grape juice other than ingestion.

MATERIALS AND METHODS

Viruses, cultures, and reactive substances

Enteroviruses used for these experiments were poliovirus type 1 (PO1) strain CHAT (obtained from the American Type Culture Collection), coxsackievirus type B3 (CB3) strain Nancy (obtained from T. G. Metcalf, University of New Hampshire), and echovirus type 6 (EC6) strain D'Amori (obtained from the American Type Culture Collection). The respiratory virus used was parainfluenza type 1, strain

Sendai (obtained from the American Type Culture Collection). Porcine enterovirus type 3, strain ECPO-6, was obtained from E. H. Bohl, Ohio Agricultural Research and Development Station.

Tissue culture lines used were Buffalo green monkey kidney (BGM; obtained from International Biological Laboratories), HeLa (obtained from Wisconsin State Laboratory of Hygiene, Madison), Vero (obtained from Flow Laboratories), and minipig kidney (MPK; obtained from the American Type Culture Collection). All cultures were passed and maintained in our laboratory by methods described previously (2).

Except as otherwise noted, PO1 and CB3 were titered on either HeLa or BGM tissue cultures using Heberling's agar overlay (2). EC6 was titered on Vero cultures using a medium based upon Eagle's MEM prepared with Hanks' solution; supplemented with nonessential amino acids, 1% whole milk, 2% fetal calf serum, 50 mM MgCl₂·6 H₂O, and 0.1% DEAE dextran; solidified with 1% Ionagar No. 2; and containing 0.0015% neutral red. ECPO-6 was titered on MPK using a solid maintenance medium based upon Eagle's MEM prepared with Earle's solution, supplemented with another 1× MEM amino acids and 5% fetal calf serum; solidified with 0.7% Noble agar; and containing 0.002% neutral red. Sendai virus was titered on BGM cells using a crystal violet staining procedure (CVF); briefly this involved incubating the cells under solid maintenance medium (Eagle's MEM + 10% fetal calf serum, solidified with 1% Noble agar) for 5-7 days, removing the agar, and then staining with crystal violet fixative (1% NaCl, 26% ethanol, 0.5% crystal violet, and 5% formaldehyde). It had been previously determined (unpublished data) that Sendai virus would not replicate in PK 15, HeLa, or Vero tissue cultures, but would replicate in either BGM or MPK tissue cultures. It had also been determined that plaques could be demonstrated by the CVF procedure but would not be produced by more conventional procedures involving neutral red in the overlay medium.

Whole, pure, heat-pasteurized Concord grape juice was supplied by Welch Foods Inc. Polyethylene glycol (PEG; m.w. 20000) was obtained from Union Carbide. Enocianina grape skin extract was supplied by K. M. Green, Coca-Cola Inc., Atlanta, GA. Feces containing coproantibody were supplied by T. E. Minor, University of Wisconsin, Madison. Human pooled blood serum was obtained from the Wisconsin State Laboratory of Hygiene.

Inactivation and reactivation

PO1, CB3, and EC6 were each inoculated into undiluted grape juice, grape juice diluted to 10%, deionized water, and glycine-HCl buffer (pH 3.3); Sendai virus was inoculated into undiluted grape juice only. All viruses were inoculated at a final level of 5×10^3 plaque-forming units (PFU)/ml. All test suspensions were held at 4 C for 4 h except Sendai virus, which was held at 4 C for approximately 18 h. After the reaction time, all solutions were titered in appropriate tissue cultures, using appropriate agar overlays.

To test the effects of temperature on inactivation, CB3 was inoculated at a final level of 5×10^3 PFU/ml into undiluted grape juice, grape juice diluted to 10%, and deionized water. Each sample was divided into three subsamples, and subsamples were reacted for 4 h at 4, 25, and 37 C. Each subsample was then titered in HeLa tissue cultures.

To test reactivation of inactivated virus by PEG, we first inoculated PO1 into both undiluted grape juice and deionized water. After

reaction for 18 h at 4 C, each sample was divided into three subsamples. One set of subsamples of both juice and water was mixed with an equal volume of 1% PEG (final concentration 0.5%), a second set of subsamples was mixed with an equal volume of 5% PEG (final concentration 2.5%), and a third set of subsamples was mixed with an equal volume of deionized water. After 1 h reaction at room temperature (ca. 25 C), all subsamples were titered using BGM tissue cultures. The same procedure was used to test for reactivation of Sendai virus, except the 5% PEG concentration was omitted.

Reactivation of inactivated virus was also tested by inoculating human blood serum with either grape juice-inactivated PO1 or unactivated PO1 contained in phosphate-buffered saline (PBS); deionized water control samples were also inoculated with both preparations of virus. After incubation for 30 min at room temperature, all samples were assayed for virus by the plaque technique on HeLa tissue culture.

Grape skin extract

Enocianina grape skin extract powder was suspended in deionized water at levels of 0.1, 1, and 5%. CB3 was incubated with these suspensions, and some of the 1% suspension was further reacted with 1% PEG, as described above. The samples were assayed in HeLa cultures by the plaque technique.

Mode of inactivation

PO1 was labelled with ^{32}P as described previously (8). To test whether the protein coat of inactivated virus had been modified so that the virus could no longer adsorb to a Millipore filter (3), we reacted labelled PO1 with both undiluted grape juice and deionized water for 4 h at 4 C. Both suspensions were then filtered through a 25-mm diameter 0.22- μm porosity Millipore GS membrane filter. To test whether the grape juice had caused aggregation of virus into more or less stable complexes, we substituted a 25-mm diameter Nuclepore polycarbonate membrane filter with a nominal pore size of 50 nm (less than two virus diameters) for the Millipore filter. Radioactivity of both filtered and unfiltered samples was counted on a Baird-Atomic GM scaler: two planchets were counted twice, an average count calculated, and the average count corrected for background.

To examine whether adsorption of inactivated virus to cells still occurred, we reacted ^{32}P -labelled PO1 with both undiluted grape juice and deionized water for 4 h at 4 C; the juice and water were then split into two subsamples. One set of subsamples was reacted for 1 h at 25 C with minipig kidney cell suspensions (MPK); this reaction was a negative control, as PO1 does not adsorb to MPK cells. The other set of subsamples was reacted for 1 h at 25 C with HeLa cell suspensions; PO1 normally will adsorb to HeLa cells. After the reaction time the cell suspensions were centrifuged for 15 min at 1000 rpm, the supernatant fluids were removed, and the packed cells were resuspended. Radioactivity of the resuspended cells, the cell supernatant fluids and suspensions not reacted with cells were counted.

Protection of digestive tract

As human enteroviruses appear to initiate their infections in the last segment of the small intestine, the ileum (I), we wanted to determine whether ingested PO1 which had been inactivated by grape juice would regain its infectivity before reaching the ileum. Untreated virus and PO1 which had been neutralized by coproantibody were included as bases for comparison. The swine digestive tract, a close analog of that of man, served as the source of the materials with which the virus preparations were treated.

PO1 was inactivated by grape juice by reacting the virus with undiluted grape juice for 18 h at 4 C. Unactivated PO1 was diluted in PBS and held for 18 h at 4 C. Coproantibody was extracted from a child's stool specimen by preparing a 20% (w/v) suspension in PBS, homogenizing for 3 min, centrifuging at 7500 rpm for 20 min, and then heating the supernatant fluid at 56 C for 30 min (9). After filtration at 0.20- μm porosity (Gelman GA-8) to remove bacteria, the supernatant fluid was reacted with PO1 and ECPO-6 for 6 h at 37 C: 99.8% of the PO1, but none of the ECPO-6, was neutralized (thus showing the presence of coproantibody). Coproantibody-neutralized PO1 was finally prepared by reacting PO1 with the coproantibody preparation

for 18 h at 4 C.

The pigs used in these experiments were obtained from a local farm when 1 day old. They were raised with littermates in a heated nursing incubator until 3½ wk old; during this time they were fed a mixture of Cadco milk replacer and powdered milk. The pigs were then held for an additional 2 wk in individual isolator cages and were fed a diet of processed cheese, powdered milk, peanut oil, eggs, oatmeal, and bananas, supplemented with vitamins and salt; they had unlimited access to water.

The first digestive tract experiment was a preliminary test of the experimental procedures. A pig that had not been fed for 24 h but that had access to water was sacrificed by electrocution. The abdominal cavity was opened, and sections of stomach, duodenum, and large intestine were tied off and removed. The sections were opened, and contents were taken from each. Deionized water control samples were also prepared. Each sample was inoculated with either grape juice-inactivated or "normal" (unactivated) PO1, and all samples were incubated for 30 min at room temperature (ca. 25 C). To remove both bacteria and intestinal contents that would interfere with culture inoculation, the samples were treated ultrasonically for 5 min, diluted 1:100 with PBS, filtered simultaneously through Miracloth (a nonwoven toweling; Chicopee Mills, New York) and a Gelman 1.2- μm -porosity (GA-4) membrane filter by positive pressure, and finally filtered by positive pressure through a Gelman 0.20- μm porosity (GA-8) membrane filter. Each sample was then assayed for virus by the plaque technique on HeLa tissue cultures.

The second digestive tract experiment was a confirmation and expansion of the first experiment. Due to watery intestinal contents in the first experiment, the pig used in the second experiment was fed 5 h before being sacrificed. Samples of six segments of the pig's digestive tract (stomach, duodenum, jejunum, ileum, cecum, and descending large intestine) were collected in the same way as for the first experiment. These and the deionized water controls were reacted with each of three different PO1 preparations: (a) virus in PBS, (b) virus inactivated by grape juice, and (c) virus neutralized by coproantibody. As before, the samples were all held for 30 min at room temperature. However, due to some problems with removal of interfering materials in the first experiment, the samples were processed somewhat differently. After the reaction time, the samples were diluted 1:100 in PBS, and were then homogenized chilled for 2 min. Each sample was then filtered in succession through Gelman membrane filters of 5.0-, 1.2-, 0.8-, and 0.45- μm porosity (GA-1, GA-3, GA-4, and GA-6, respectively). The 5.0- μm filter was 47 mm in diameter, and filtration was done by vacuum; the other filters were 25 mm in diameter, and filtration was done by positive pressure. Finally, each sample was filtered by positive pressure through a Gelman 25-mm diameter 0.20- μm porosity (GA-8) membrane filter. Although there was still some extraneous material left, this was a great improvement over the procedure used in the first experiment. The samples were then titered as previously described.

RESULTS

Inactivation and reactivation

There was no difference between the acid buffer and the deionized water (pH approx. 7.0) results for any of the three viruses tested (Table 1); thus acidity is not a cause of the inactivation phenomenon. More than 99% of both PO1 and EC6 was inactivated in both undiluted and 10% grape juice. CB3 showed >99% inactivation in undiluted grape juice, but only 76% of CB3 was inactivated by a 10% grape juice solution.

There was little difference between samples at different temperatures for CB3 virus (Table 2), and the results of the 4 C samples closely agreed with the results obtained in the first CB3 experiment (Table 1). Thus the temperatures tested were not a factor in viral inactivation of grape juice.

TABLE 1. *Inactivation of enteroviruses by grape juice.*

Sample suspension	Virus		
	PO1	CB3	EC6
Grape juice, undiluted	0 ^a	0	0
Grape juice, diluted to 10%	0	21	0
Deionized water	84	88	71
pH 3.3 buffer	86	93	70

^aTotal of plaques recorded among four culture flasks per sample suspension.

TABLE 2. *Effect of temperature upon inactivation of CB3 virus by grape juice.*

Sample suspension	Temperature		
	4 C	25 C	37 C
Grape juice, undiluted	0 ^a	0	0
Grape juice, diluted to 10%	21	20	19
Deionized water	83	93	90

^aTotal of plaques recorded among four culture flasks per sample suspension.

Reactivation of inactivated PO1 by PEG is shown in Table 3. Of the inactivated virus, 61% was reactivated by 0.5% PEG; about the same amount of virus was reactivated by 2.5% PEG (68%). Thus most of the virus is not irreversibly inactivated by grape juice; furthermore, at PEG concentrations tested, an increase of PEG does not cause additional reactivation of virus.

Results of both inactivation and reactivation of Sendai virus are in Table 4. This was the first tested virus against which grape juice was virtually ineffective (14% inactivation); others (Table 1) had undergone >99% inactivation under similar conditions. The small grape juice effect was completely reversed by PEG.

The results of the reactivation experiment with human blood serum are shown in Table 5. As can be seen from comparing these results with those in Table 3, human blood serum causes greater reactivation ($\geq 94\%$) than PEG of PO1. Given this degree of reactivation, it is clear that the antiviral substances, which would presumably occur in the serum if present in the blood, could not be detected simply by adding virus to the blood. Other means of detecting these substances in

TABLE 3. *Reactivation by PEG of grape juice-inactivated PO1 virus.*

Sample suspension	PEG concentration(%)		
	0	0.5	2.5
Grape juice, undiluted	0 ^a	51	61
Deionized water	83	84	90

^aTotal of plaques recorded among four culture flasks per sample suspension.

TABLE 4. *Inactivation by grape juice and reactivation with PEG of Sendai virus.*

Sample suspension	PEG concentration (%)	
	0	0.5 ^a
Grape juice, undiluted	82 ^b	57
Deionized water	95	51

^aAdditional two-fold dilution of virus suspension, as compared with 0 PEG concentration.

^bTotal of plaques recorded among four culture flasks per sample suspension.

TABLE 5. *Reactivation by human blood serum of grape juice-inactivated PO1 virus.*

Sample suspension	Reactivation		
	None	Deionized water	Serum
Grape juice, undiluted	0 ^a	0	88
PBS	99	102	94

^aTotal of plaques recorded among four culture flasks per sample suspension.

blood could only be undertaken if the nature of the substances were more precisely known.

Grape skin extract

Results of the experiments with grape skin extract are in Table 6. It is difficult to find a valid basis on which to compare these results with those reported above for virus inactivation by grape juice because there is no convenient conversion factor by which to relate the skin extract to the whole grape juice. Although each of the suspensions of skin extract inactivated substantially more CB3 than did the 10% solution of grape juice reported above (93.9% for 0.1% skin extract vs. 78% for 10% juice), the degree of reactivation by PEG was essentially the same as was seen with virus inactivated by grape juice.

TABLE 6. *Inactivation by grape skin extract and reactivation with PEG of CB3 virus.*

Effect tested	Grape skin extract (%)	Virus titer (PFU/ml)	Effect (%)
Inactivation (no PEG)	0	4100	—
	0.1	250	93.90
	1	4	99.90
	5	1	99.98
Reactivation (0.5% PEG) ^a	0	2600	—
	1	1600	62

^aAdditional two-fold dilution of virus suspension, relative to those with no PEG.

Mode of inactivation

More than 95% of ³²P-labelled PO1 was adsorbed during filtration through a 0.22 μ m porosity Millipore GS membrane filter, either before (1123 CPM/ml vs. 52 CPM/ml) or after (1233 CPM/ml vs. 55 CPM/ml) inactivation by grape juice. This adsorption is a property of the coat protein surface of the virus particle, which evidently is not modified greatly by grape juice inactivation.

Essentially none of the ³²P-labelled PO1 was retained by a 50-nm porosity Nuclepore filter, either before (1050 CPM/ml vs. 1131 CPM/ml) or after (1254 CPM/ml vs. 1236 CPM/ml) inactivation by grape juice. Thus aggregation is not what the grape juice does to the virus.

The results of the cell adsorption studies are in Table 7. As expected, there was no adsorption of either inactivated or "normal" virus to MPK cells (97% of radioactivity remained in the supernatant fluid). "Normal" virus adsorbed to the HeLa cells somewhat better than expected (82%). However, there was significant adsorption of inactivated virus to HeLa cells (17%). Although the adsorption was much lower than that of "normal" virus, it was significantly higher than that of the negative (MPK) controls. These results

TABLE 7. Uptake of grape juice-inactivated P01 by cells.

Sample suspension	Cells	Fraction	
		Supernatant fluid (CPM/ml)	Resuspended cells (CPM/ml)
Grape juice, undiluted	None	599	—
	MPK	540	12
	HeLa	568	120
Deionized water	None	599	—
	MPK	569	18
	HeLa	131	585

indicate that, whereas most of inactivated virus is blocked from cell receptor sites by the active substances in grape juice, ca. 20% of the virus remains able to adsorb. It is unknown why this 20% fails to express itself in plaques.

Protection of digestive tract

The data for the experiments involving reactivation of inactivated and neutralized virus by contents of the digestive tract are in Table 8. Reactivation of virus inactivated by grape juice occurred in all samples (except the deionized water control) and ranged from 42 to 82%. Although reactivation of coproantibody-neutralized virus did not take place in contents of jejunum, ileum, or large intestine, it would be expected that virus reactivated in the stomach or duodenum would infect upon reaching the ileum. Thus the data show that virus inactivated by grape juice or neutralized by coproantibody would still present a health hazard due to reactivation in the intestinal tract.

DISCUSSION

The findings of Konowalchuk and Speirs (5,6,7) have been confirmed. We worked only with Concord grape juice, but they found the virus inactivation phenomenon to be a generic property of grapes and grape products. They reported that enteroviruses, as well as members of other virus groups which we did not test, are inactivated by grape juice. We also found that the Sendai strain of parainfluenza virus type 1, which they did not test, is virtually refractory to the effect of grape juice.

The loss of infectivity titer that occurs when

enteroviruses are treated with grape juice is neither the result of aggregation nor of modification of the particle's surface sufficient to interfere with its nonspecific adsorption to a cellulose nitrate membrane filter. In fact, a portion of the treated virus adsorbs specifically to host cell receptors, even though it cannot then initiate a replicative cycle.

The finding that most of the virus can be reactivated by PEG and other substances makes it clear that the particle is not being degraded in the process of inactivation. The active substances are said by Konowalchuk and Speirs (5,6) to be polyphenols of low to moderate molecular weight, derived principally from the skin of the grape. They tested several pure compounds of this class and showed them to inactivate enteroviruses.

We have tried to assess the significance of this phenomenon to human health. We wished to learn whether ingested grape juice was likely to be able to prevent or modify virus infections in man. The experimental approaches were indirect, in that no human subjects were used, but were generally pertinent to this question.

Because grape juice is a food, it seemed most reasonable to look first for protection of the digestive tract. Contents of various portions of the digestive tract were obtained from young swine: their digestive tract is quite like that of man, and they had been fed on human foods to heighten the analogy. Under conditions which we think are representative of those inside the digestive tract, extensive virus reactivation took place with contents from each segment. Coproantibody-neutralized virus was included because the neutralization process is also known to be reversible and not to degrade the virus. Reactivation of coproantibody-neutralized virus differed in some specifics, but it was clear that neither coproantibody nor grape juice was likely to prevent enterovirus infection of the human intestines.

If ingested grape juice were going to act against viral infections at other sites in the body, the active substances would probably have to be absorbed from the digestive tract and transported via the bloodstream to these

TABLE 8. Reactivation of grape juice-inactivated and coproantibody-neutralized P01 by contents of pigs' digestive tracts.

Tested suspension	Experiment number	Virus treated with				
		PBS	Grape juice		Coproantibody	
		Plaque count	Plaque count	Reactivation (%)	Plaque count	Reactivation (%)
Input	1	96 ^a	0	—	—	—
	2	94	0	—	0	—
Stomach	1	92	50	54	—	—
	2	96	48	50	48	50
Duodenum	1	101	56	55	—	—
	2	99	59	60	41	41
Jejunum	2	98	50	51	0	<1
Ileum	2	95	55	58	0	<1
Cecum	2	103	43	42	81	79
Large intestine	1	88	72	82	—	—
	2	96	70	73	0	<1
Deionized water	1	96	0	<1	—	—
	2	96	0	<1	0	<1

^aTotal among four flasks per sample suspension.

alternate locations. The straightforward approach to detecting these substances in blood, direct testing with virus, proved not to be feasible because blood serum reactivates inactivated virus with unequal efficiency. Any other approach to detecting these substances in transit in the blood would require much more exact knowledge of their nature than now exists.

Sendai virus was selected as a potential model virus because it produces lethal respiratory infections in mice under controlled experimental conditions (4). The question of whether ingestion of grape juice could produce effective levels of active substances in the murine respiratory tract was mooted by the inability of grape juice to inactivate the Sendai virus.

These laboratory experimental approaches have failed to produce evidence that ingested grape juice would be valuable for antiviral prophylaxis or therapy in man. We think the potential purchaser would do best to continue to judge the product on its merits as a food.

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Drug Residues in Dairy Culls Target of New USDA Program

Dairymen should keep up-to-date records on drugs they administer to dairy cows. If they don't, they can be asking for trouble when they cull antibiotic treated cows.

A U.S. Department of Agriculture program to detect drug residues in cull animals sold for slaughter goes into effect early in 1979. Under the "swab test on premises" (STOP) program, dairymen can be fined or can face charges if the cows they sell are found to contain illegal levels of drug residues in their tissues. Federal meat inspectors will be using a new detection technique to find drug residues before carcasses leave the slaughterhouse.

"Dairymen must read drug labels, keep track of the dates they administer medications and not sell cattle before the withdrawal time indicated on the label," says Allan N. Bringe, University of Wisconsin-Extension dairy scientist. "The dairyman is responsible for cull cows marketed being free of residues."

The USDA estimates that about 7,000 cows going to market each year contain residues of drugs used by farmers to treat various illnesses and injuries, including mastitis. The department doesn't object to farmers marketing animals that have been treated or that have disorders, but it does insist that they market only when the animals are free of residues.

The drugs most frequently found in illegally high amounts are the antibiotics, penicillin and dihydro-streptomycin.

One effect of carelessness in drug use is lower prices for cull cows. Carcasses in which high levels of residues are found must be condemned. Current cull cow prices are in the \$30-\$40 per hundredweight range.

Bringe says the old test used to detect residues took 14 days to confirm specific residues. A new swab test technique will find antibiotic residues in an animal's kidneys within a few hours. If residues are found, further lab tests will be carried out. If the level exceeds U.S. Food and Drug Administration (FDA) tolerances, the carcass will be

condemned.

"The risk for dairymen who send cull animals to market in violation of residue tolerances is now much higher," says Bringe. "It won't take long for packers to learn which producers or animal dealers are marketing cows with violative levels. Packers may refuse to buy animals from individuals who consistently violate the law."

Under the new program, federal inspectors will report incidents of violation to the FDA, which has the authority to investigate and take action. If problems continue, new and tougher laws on antibiotic use could be imposed.

Bringe urges dairymen to review their present use of drugs for disease prevention and control. "Ask yourself if the drugs are really needed. If you're convinced they are, be sure to follow proper procedures and insist those who work with your animals also follow them. Buy medications from reputable suppliers and sell animals to reputable dealers.

"Good records should have the highest priority," says Bringe. "Keep track of what dose was given to which

con't p. 109