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Cranberry juice constituents affect influenza virus adhesion and infectivity

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Abstract

Cranberry juice contains high molecular weight materials (NDM) that inhibit bacterial adhesion to host cells as well as the co-aggregation of many oral bacteria. Because of its broad-spectrum activity, we investigated NDM's potential for inhibiting influenza virus adhesion to cells, and subsequent infectivity. Hemagglutination (HA) of red blood cells (RBC) caused by representatives of both influenza virus A subtypes (H_1N_1 and H_3N_2) and the B type was inhibited by NDM at concentrations of 125 μg/ml or lower, which is at least 20-fold lower than that usually found in cranberry juice. A dose–response effect of NDM on HA was demonstrated. The infectivity of the A and B types was significantly reduced by preincubation with NDM (250 μg/ml), as reflected by the lack of cytopathic effect on Madine-Darby canine kidney (MDCK) cells and the lack of HA activity in the media of infected cells. The effect of NDM was also tested after A or B type viruses were allowed to adsorb to and penetrate the cells. Various levels of reduction in virus tissue culture infective dose $TCID_{50}$ were observed. The effect was most pronounced when NDM was added several times to the infected MDCK cells. Our cumulative findings indicate that the inhibitory effect of NDM on influenza virus adhesion and infectivity may have a therapeutic potential.

Keywords: Influenza; Cranberry; NDM; Antiviral effect

Influenza, a highly communicable acute respiratory disease, predisposes to a number of complications, resulting in a severe worldwide economic burden. Prevention and control of both the annual influenza epidemics and its infrequent but severe pandemic outbreaks are achieved by the use of vaccines and newly emerging antiviral drugs.

These vaccines provide sometimes lower than desirable protection, particularly in the immunocompromised and the elderly, the two most susceptible subpopulations (Keren et al., 1988; Admon et al., 1997). Furthermore, the vaccines currently available are designated for intramuscular injection, resulting mainly in serum antibodies. It follows that a negligible amount of mucosal antibodies are present at the access site of the virus. In addition, vaccines are generally unavailable in the early stages of a pandemic (WHO, 1999) and antiviral drugs may be the only means of intervention.

Two classes of antiviral drugs are used:

(i) Anti-M2 inhibitors amantadine and rimantadine, effective against A strains only (WHO, 1980). A reduction in the severity and duration of the signs and symptoms is recorded when they are administered within 48 h of disease onset (ACIP, 1996).
(ii) Neuraminadase inhibitors, effective against both A and B viruses and better tolerated than the former. To date, strains resistant to the drugs are not clinically important, probably because they are not as virulent as the parental strains. As prophylactics, these inhibitors are 70–90% effective and may shorten the duration of illness by 1.5 days when used within the first 48 h (Treanor and Falsey, 1999; Hayden et al., 1999). Therefore, there is a need

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for novel approaches and a new class of substances to prevent and contain influenza outbreaks.

Physicians have long recommended consumption of cranberry juice to avoid urinary tract infections. It was hypothesized that the prevention of such infections is due to the inhibition of *E. coli* adhesion to uroepithelial cells by cranberry constituents (Ofek et al., 1991). Studies have shown that cranberries contain high and low molecular weight constituents [nondialyzable material (NDM) and proanthocyanidins, respectively], which act in vitro to inhibit the adhesion of diverse microbial species (Ahuja et al., 1998; Burger et al., 2000; Foo et al., 2000; Weiss et al., 1998; Zafriri et al., 1989). Although it has been suggested that cranberry proanthocyanidins are one of the active anti-adhesion agents (Foo et al., 2000), we found that NDM was at least six times more active (weight per volume) than the cranberry proanthocyanidins in inhibiting bacterial adhesion and co-aggregation of various representative bacteria (unpublished data).

Since NDM exhibits a broad anti-adhesion activity, it was of interest to determine whether it inhibits the attachment and subsequent replication of influenza viruses. In the present study we addressed this issue by testing the activity of NDM on influenza virus-mediated red blood cell (RBC) hemagglutination (HA) as well as on in vitro replication of the virus.

Cranberry juice from the American cranberry, *Vaccinium macrocarpon*, and a proanthocyanidin-rich fraction were obtained from Ocean Spray, Inc. The juice was dialyzed at 4 °C for 10 days against distilled water, changed 10 times, in dialysis bags of 15,000 MW cut-off and lyophilized. The nondialyzable material exhibits tannin-like properties, is highly soluble in water, devoid of proteins, carbohydrates and fatty acids and contains 56.6% carbon and 4.14% hydrogen (Ofek et al., 1996). As opposed to proanthocyanidins, the chemical structure of NDM is not well defined, owing to its high molecular weight (>15,000) and because attempts to degrade it by conventional chemical means into lower-sized molecules have proved unsuccessful (unpublished data). Therefore, also no information was obtained using nuclear magnetic resonance (NMR) as well as matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) mass spectrometry and chromatographic procedures to resolve its structure.

The following influenza virus strains were used:

1. A/PR/8/34 H_1N_1 grown in the allantoic sac of 10–11-day-old eggs to a 1:1024–2048 HA titer, or grown in Madine-Darby canine kidney (MDCK) cells to a 1:128 HA titer.
2. A/H_1N_1 (1:128 HA titer) and A/H_3N_2 (1:256 HA titer), clinical isolates grown in MDCK cells.
3. A/Panama 2007/99 H_3N_2 adapted to MDCK cells (1:256 HA titer).
4. B/Yamanashi/166/98, grown in MDCK cells (1:256–512 HA titer). To evaluate replication inhibition, viruses cultivated in MDCK cells were used.

Table 1
Effect of cranberry juice constituents (NDM) on viral hemagglutination and replication in MDCK cells

Viral strain	HAU[a]	MDCK cells (log_{10})[b]		
	Nontreated	Treated	Nontreated	Treated
A/PR8/34				
Egg	16	<1	NP	NP
MDCK	16	2–4	6.5	1.5[c]
A/H_3N_2	16	4–8	6.5	1.5[c]
A/H_1N_1	16	4–8	6.0	1.5[c]
B/Yamanashi	16	<1	7.5	1.5[c]

[a] Hemagglutination units of treated virus (preincubated with 125 μg/ml of NDM), compared with those of nontreated virus.
[b] $TCID_{50}$ in MDCK cells of treated virus (preincubated with 250 μg/ml of NDM), compared with that of nontreated virus.
[c] Significantly different from the corresponding nontreated virus ($p < 0.001$, Fisher's Exact Test). Each experiment was repeated at least twice.

To determine HA, 0.1 ml of twofold dilutions of each virus suspension in phosphate-buffered-saline (PBS) was mixed with 0.1 ml of a 0.5% chicken RBC or 1% sheep RBC or human 1% O RBC suspension and scored after 30 min incubation at room temperature (Sever, 1962).

At 500 μg/ml, NDM did not cause hemolysis or spontaneous HA of any of the RBC tested. The HA data are summarized in Table 1. Preincubation of NDM (125 μg/ml) with A/PR/8 (egg-grown) or B/Yamanashi (grown in MDCK cells) strains inhibited virus-induced HA. This was reduced from 16 HA units (HAU) to <1 in NDM-containing virus suspensions (100%). NDM at 125 μg/ml reduced the 16 HAU of the two clinical isolates A/H_1N_1 and A/H_3N_2 to 4–8 units. A/PR/8 grown in MDCK cells was less sensitive to 125 μg/ml NDM; the 16 HAU were reduced to 2–4 HAU (Table 1). A higher NDM concentration (400 μg/ml) was needed to reduce the HAU of the MDCK cell-grown A/PR/8 strain from 16 to <1 units (not shown). Chess board titration of virus densities and decreasing NDM concentrations revealed that as little as 4, 16, 64 and 128 μg/ml NDM were needed to completely inhibit HA induced by 8, 16, 32, 64, respectively, HAU of virus A/PR8/34. The results indicate a highly significant $r^2 = 0.980$ correlation between HAU and NDM concentration required to completely inhibit HA (Fig. 1). HA inhibition was also observed using sheep or human RBC, consistent with the notion that the target for NDM is the virus.

To test inhibition of virus replication, MDCK cells were grown in DMEM medium supplemented with 10% inactivated FCS and antibiotics (100 μg/ml penicillin G and 100 μg/ml streptomycin). For the assay, cells were grown in 12-well culture plates (Nunc, Roskilde, Denmark) in a humidified atmosphere with 5% CO_2 at 37 °C, and used when confluent monolayers had formed (48 h). NDM inhibition of virus infectivity in vitro was tested by incubating 250 μg/ml NDM with virus for 2 h at room temperature. Tenfold serial dilutions in medium without serum were inoculated in triplicate wells containing MDCK monolayers. The virus was allowed to adsorb for 1 h at 37 °C. The inoculum was removed and the medium was supplemented with 1% serum

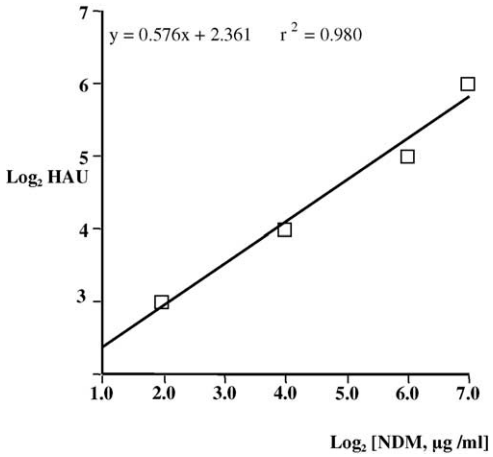

Fig. 1. NDM concentration causing 100% inhibition of virus HA activity. Each of the virus titers (A/PR8/34), expressed as $\log_2$ of HAU, was incubated with twofold serial dilutions of NDM in order to determine the minimal concentration required for complete inhibition of virus HA. Each experiment was performed in triplicate.

and trypsin, 2 μg/ml (2× crystallized), was added. Following 4 days incubation at 37 °C in a 5% CO_2 incubator, the monolayers were examined for cytopathic effect (CPE) (Fig. 2) and the respective supernatants were assayed for HA. HA was evident at a dilution of up to 10^{-7} in the control wells, whereas in wells infected with the virus-NDM suspension, hemagglutination was observed only at 10^{-1}. For the CPE, the monolayers were washed with PBS to remove dead cells and debris, fixed with cold methanol and stained with crystal violet to determine the integrity of the monolayer. The CPE was observed in all the control wells (Fig. 2 rows A, B and C) at a 10^{-7} viral dilution whereas in the virus-NDM suspension the CPE was observed at 10^{-1} (Fig. 1 rows D, E and F). The observed CPE correlated with the HA results (not shown), allowing calculation of tissue culture infective dose ($TCID_{50}$, Reed and Muench, 1938). NDM reduced the B/Yamanashi titer from $10^{7.5}$ to $10^{1.5}$ (Table 1). Significant

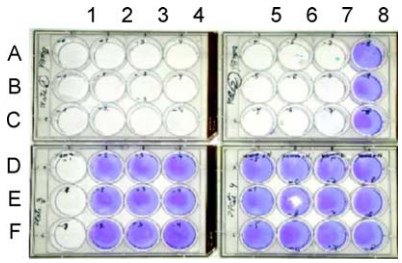

Fig. 2. Prevention of virus-induced cytopathic effect by NDM. MDCK monolayers were inoculated in triplicate with 10-fold dilutions (columns 1 to 8) of a B/Yamanashi virus suspension (rows A, B and C) or with a mixture of NDM (250 μg/ml) and B/Yamanashi virus, in triplicate (rows D, E and F). Following 1 h adsorption, the inoculum was removed, fresh medium was added and the cultures were incubated for 4 days. The monolayers were then fixed and stained for CPE. Blue staining indicates that the cells are intact; staining is not obtained if the cells are destroyed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

inhibition of infectivity by NDM was demonstrated for all the virus strains tested ($p < 0.001$, Fisher's Exact Test): the A/H_3N_2 was reduced from $10^{6.5}$ to $10^{1.5}$, A/H_1N_1 from $10^{6.0}$ to $10^{1.5}$ and A/PR8 (grown in MDCK cells) from $10^{6.5}$ to $10^{1.5}$ (Table 1). When A/H_3N_2 and NDM were added simultaneously, $TCID_{50}$ dropped from $10^{6.5}$ to $10^{2.0}$ and from $10^{6.0}$ to $10^{1.5}$ when A/PR8 was tested. These data suggest that the NDM-virus interaction was virtually instantaneous and that preincubation is not required. Inhibition of infectivity in MDCK cells was similar for the A strains (4.5–5 logs), B/Yamanashi appeared to be more sensitive (6 logs).

Although in our previous studies we observed very little batch-to-batch variation in the ability of NDM to inhibit bacterial adhesion, further experiments are required to confirm such variations in the virus system, as all experiments in this study were performed with one batch of NDM.

To evaluate the potential of the NDM in the therapy of viral infection, the cells were first exposed to viral suspensions to allow adsorption followed by penetration into the cells for 1 h. NDM (100 μg/ml) was added at various post-infection time intervals as indicated (Table 2). Viral $TCID_{50}$ was determined in the treated cultures following 4 days and 6 days incubation by assaying the supernatants for HA activity, as described above. The results show that NDM reduced the virus $TCID_{50}$ for the entire time of the follow-up (6 days post-infection). This effect was most accentuated when the NDM was added several times to the infected MDCK monolayer (Table 2). These results demonstrate that NDM can reduce or inhibit virus replication indicating inactivation of newly formed virus.

We also compared the effect of cranberry proanthocyanidins with that of NDM. A four- to fivefold higher proanthocyanidin concentration was required to completely inhibit 16 HA units of A/PR8 (grown in egg), confirming our previous observation that NDM is significantly more potent than cranberry proanthocyanidins (unpublished data).

To be clinically effective, an antiviral drug should prevent infection, arrest viral growth, eliminate the virus from the cell or suppress viral multiplication, allowing an effective host reaction (WHO, 1969). However, the use of the currently available antiviral drugs such as anti-M2 inhibitors and neu-

Table 2
Effect of NDM added after infection on virus replication in MDCK cells

Viral strain	Control	Time (h) of NDM treatment post-viral adsorption[a]			
		1	6	24	1 + 6 + 24
A/Panama H3N2	3.5/3.5	<1/3	1.5/2.5	2/2.5[b]	<1/<1[c]
B/Yamanashi	4.5/4.5	2.5/3	2/3[b]	3/>3[b]	<1/1.5[c]

The numbers indicate the viral $\log_{10}$ $TCID_{50}$, determined following 4/6 days incubation by assaying the supernatants for HA as described in the text. The values are the averages of triplicates of two independent experiments.

[a] NDM, 100 μg/ml, was added in each treatment.

[b] Logistic regression at a confidence interval of 95% yielded odds of 42–91.

[c] Logistic regression at a confidence interval of 95% yielded odds of ≥2110.

raminadase inhibitors is limited, owing to their low effectiveness, strain specificity, side effects and the emergence of resistant strains (Hayden et al., 1991). Since NDM is prepared from cranberries and its antiviral effect was demonstrated at concentrations lower than that found in cranberry juice cocktail, it is unlikely to cause any significant side effects. NDM may belong to a class of natural antiviral substances. It appears that NDM interacts directly with the virus, as pretreatment of MDCK cells or RBC with NDM followed by rinsing, had little or no effect on the virus-induced HA or $TCID_{50}$. NDM probably exerts its effect by preventing viral adsorption onto the cells because it inhibited viral HA, which is mediated by the sialic acid-specific hemagglutinin. Moreover, its effect on the infectivity of the virus was more pronounced when added several times post-infection, suggesting that it prevented the adsorption of viral progeny released by infected cells onto new cells. Based on our cumulative findings, it is possible that NDM could be used as an aerosol or for intranasal administration to control influenza viral infection. The anti-bacterial adhesion effects of NDM on bacterial adhesion and aggregation might have an important added value in preventing bacterial infections secondary to viral ones.

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