

Suppression of influenza A virus nuclear antigen production and neuraminidase activity by a nutrient mixture containing ascorbic acid, green tea extract and amino acids

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Abstract. Influenza, one of the oldest and most common infections, poses a serious health problem causing significant morbidity and mortality, and imposing substantial economic costs. The efficacy of current drugs is limited and improved therapies are needed. A unique nutrient mixture (NM), containing ascorbic acid, green tea extract, lysine, proline, N-acetyl cysteine, selenium among other micronutrients, has been shown to exert anti-carcinogenic and anti-atherogenic activity both *in vitro* and *in vivo*. Many of the constituents of NM have been shown to have an inhibitory effect on replication of influenza virus and HIV. This prompted us to study the effect of NM on influenza A virus multiplication in infected cells and neuraminidase activity (NA) in virus particles. Addition of NM to Vero or MDCK cells post infection resulted in dose-dependent inhibition of viral nucleoprotein (NP) production in infected cells. NM-mediated inhibition of viral NP was selective and not due to cytotoxicity towards host cells. This antiviral effect was enhanced by pretreatment of virus with the nutrient mixture. Individual components of NM, namely ascorbic acid and green tea extract, also blocked viral NP production, conferring enhanced inhibition when tested in combination. Incubation of cell-free virus with NM resulted in dose-dependent inhibition of associated NA enzyme activity. In conclusion, the nutrient mixture exerts an antiviral effect against influenza A virus by lowering viral protein production in infected cells and diminishing viral enzymatic activity in cell-free particles.

Keywords: Influenza virus, nutrients, selective antiviral effect, NP viral antigen, neuraminidase activity

1. Introduction

Influenza, a febrile, acute viral disease of the respiratory tract, is one of the oldest and most common infections, affecting 20% of the world's population [4]. It poses a serious health problem causing significant morbidity and mortality, and imposes substantial economic costs. Annual influenza infections

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in the United States occur as epidemics each winter (usually December to May) and affect 5% to 20% of the population, resulting in an estimated 36,000 deaths and greater than 200,000 hospitalized patients each year [4].

Recent events, such as avian flu-associated human deaths, have caused concern over the possibility of pandemics. Although vaccination is the primary strategy for prevention of influenza, especially in high-risk populations, vaccines are often ineffective, due to antigenic drift in the virus after formulation of the current year's vaccine, rendering it less effective. Therefore, therapeutic focus has shifted to antiviral agents as part of a rational approach to controlling the epidemic of influenza.

Currently two classes of drugs are approved for the treatment or prophylaxis of influenza infections: the adamantanes (amantadine and its derivative rimantadine) and the newer neuraminidase (NA) inhibitors (oseltamivir and zanamivir). However, current therapies are not optimal and improvements are needed. Adamantanes interfere with viral uncoating inside the cell and are effective only against influenza A. They are associated with several toxic side effects and with drug-resistant variants [21]. Neuraminidase inhibitors such as oseltamivir and zanamivir, block the function of neuraminidase protein, preventing the release of virus from the host cell surface. Although neuraminidase inhibitors are effective against all strains of influenza, they must be administered early since replication of influenza virus in the respiratory tract reaches its peak between 24–72 hours after the onset of the disease. Tamiflu (oseltamivir), a neuraminidase inhibitor has been reported to reduce illness by 1–2 days and to have few adverse effects (nausea, vomiting, abdominal pain in 5%). Tamiflu does not prevent the flu but is reported to reduce the length and severity of symptoms [21]. However, recent reports on the use of the drug in normal flu cases in Japan, a country where Tamiflu prescription is ten times that in the US, reported bizarre psychiatric problems in children treated with the drug, leading to death [20]. In addition, in a recent study in Japan, 9 out of 50 treated children harbored viruses with mutations in the neuraminidase gene that encoded drug-resistant neuraminidase proteins, a serious concern since children are an important source of the spread of influenza in the community [21]. More recently FDA has recommended new warnings about possible dangerous psychiatric side effects of influenza drugs Tamiflu and Relenza. Other approaches in the field of influenza antiviral research, including RNA viral interference through the use of short interfering RNA (siRNA) or microRNA are still at an experimental stage of development [7,13].

Our approach has been focused on developing strategies to inhibit viral infections and other diseases by naturally occurring nutrients. Our previous research with cancer and heart disease has demonstrated that a nutrient mixture (NM) containing lysine, proline, ascorbic acid, green tea extract, N-acetyl cysteine (NAC), selenium and other micro nutrients has potent anti-carcinogenic and anti-atherogenic activity both *in vitro* and *in vivo* [16,27]. Since some viruses can spread in the body using similar mechanisms as cancer, such as production of MMPs in influenza virus-infected cells as demonstrated by Yeo et al. [32], we postulated that this relatively non-toxic nutrient mixture would have an effect on influenza viral infection in host cells.

Many of the constituents of NM have been reported in the literature to have an inhibitory effect on replication of influenza virus and other viruses [2,6,10,14,15,22,29,30]. This prompted us to study the effect of NM and its specific components, such as ascorbic acid (AA) and green tea extract on human influenza A virus, subtype H1N1.

The choice of the cell line is critical for the influenza virus to grow. Moreover the cell line should be easily maintained. The Madin-Darby canine kidney (MDCK) cell line has long been used as a permissive host cell type to grow the virus successfully. Vero cells are also used as another well-studied cell line for this purpose [32]. Hence, in this antiviral investigation, both cell lines were utilized. Specifically, we investigated the effect of the nutrient mixture and its key components on viral nuclear protein (NP) production in influenza A-infected MDCK and Vero cells. Additionally, we evaluated the effect of NM on virus-associated neuraminidase activity in cell-free particles that is linked to infectivity.

2. Methods and materials

2.1. Composition of nutrient mixture (NM)

For experiments, a stock solution of 1 mg/ml of the nutrient mixture was prepared by dissolving in 10 ml of serum-free Eagle's MEM and sterilized by passing through a 0.2 μm filter. NM stock solution was composed of the following in the ratio indicated: Vitamin C (25.5% as ascorbic acid, 24.8% as calcium ascorbate, 24.8% as magnesium ascorbate and 24.8% as palmitoyl ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 μg ; copper 2 mg; manganese 1mg. Individual components, namely ascorbic acid and green tea extract powder, were obtained from Vita Tech International. In regard to Vitamin C components, magnesium and calcium ascorbate are water-soluble salts while palmitoyl ascorbate is lipid soluble, enabling absorption into cell membranes.

2.2. Cell culture and treatments

Madin-Darby canine kidney (MDCK) cells and Vero cells were obtained from ATCC (American Type Culture Collection, Rockville, MD), and were grown in Eagle's MEM (EMEM, containing non-essential amino acids with L-glutamine), supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in 75 cm² tissue culture flasks (Costar, Cambridge, MA). Cells were incubated with growth media at 37°C in a humidified tissue-culture incubator equilibrated with 95% air and 5% CO₂. At near confluence, uninfected or infected cells in 24-well or 96-well plates were treated with various concentrations of the nutrient mixture (NM) or its component(s), dissolved in media and tested in triplicate at each dose. The pH of the NM, ascorbic acid or green tea (GT) solutions applied to cells were in the physiological range. The plates were returned to the incubator and, at defined time interval, cultures were assessed for viability, cytotoxicity or production of viral NP antigen. Culture media components were purchased from Gibco (Invitrogen Corp, Carlsbad, CA).

2.3. Viability assays

Cell viability was assessed by the trypan blue dye exclusion test. MDCK or Vero cells were treated with the test reagents in triplicate at each concentration and, after 48 h, cells were trypsinized, an aliquot of the cell suspension was mixed with one-tenth volume of 0.4% trypan blue and the number of cells which excluded the dye were counted in a haemocytometer. Viable cell count was expressed as the number of dye-excluding cells per ml of cell suspension.

2.4. Cytotoxicity assays

Cytotoxicity was determined by spectrophotometric measurement of total biomass by staining cellular proteins with the Sulforhodamine B dye as described by Skehan et al. [28]. Briefly, MDCK or Vero cells were treated with the test reagents in replicate at each concentration and, after 24 or 48 h, cells were washed, fixed with 50% trichloroacetic acid, rinsed, air-dried and stained with Sulforhodamine B. The incorporated dye was liberated from cells in a Tris base solution and the total protein (biomass) was determined by measuring absorbance at a wavelength of 565 nm. The degree of cytotoxicity by test reagent was the change in absorbance relative to untreated control culture.

2.5. *Effect of NM and ascorbic acid on influenza A nuclear protein (NP) in Vero cells*

Two-day old, sub-confluent monolayers of Vero cells growing in 96 or 24-well plates at density indicated (~ 20,000 cells/well or 50,000 cells/well respectively) were washed with Dulbecco's PBS (D-PBS) and exposed to human influenza A virus, (H1N1) TC-adapted (strain A/WSN/33 from ATCC) in serum-free medium (25 μ L/well or 50 μ L/well of diluted virus, respectively) at a multiplicity of infection (MOI) of 1, at 33°C for one hour. The virus inoculum was left in and cells were fed serum-free medium (without or containing 1.0 μ g/ml TPCK-treated trypsin, as indicated) supplemented with various concentrations of NM or ascorbic acid, as indicated above, in triplicate, and incubated further at 33°C. After 44 h, an equal volume of lysing solution (from Takara kit) was added to each well and an aliquot of the total lysate was assayed for the level of influenza A nuclear protein (NP) along with positive control (standards) using an enzyme-linked immunoassay (ELISA) kit (MK120 from Takara Mirus Bio Corp., Madison WI). The data were plotted to show graphically the amount of influenza A virus antigen (units/ml) detected in total lysate (infected cells plus supernatant) of cultures treated for 48 h (on Y-axis) as a function of various doses of NM (on X-axis).

2.6. *Effect of NM on influenza A nuclear protein (NP) in MDCK cells*

The protocol for evaluation of virus growth in MDCK cells was similar to that in Vero cells, as described above. To assess the influence of cell pretreatment with nutrient mixture, sub-confluent MDCK monolayers prepared as above were washed with D-PBS and treated with indicated concentrations of NM or no NM in serum-free EMEM medium (containing 0.125% BSA) at 33°C overnight. The following day, NM was removed by washing the cells twice with D-PBS followed by exposure to untreated or mock-treated virus at MOI of 1 at 33°C for 2 h. The virus inoculum was removed; the cells were washed and incubated in serum-free EMEM supplemented with TPCK-treated trypsin. After 30 h, lysates were prepared and assayed for the influenza NP antigen using the MK 120 ELISA kit.

To assess the influence of virus pretreatment with nutrient mixture, influenza A virus, subtype H1N1 (A/WSN/33 from ATCC) was pretreated with indicated final concentrations of NM or mock treated (no NM) at 33°C for 1h and placed on ice. Two-day old, sub-confluent monolayers of MDCK cells in 96-well plate (~ 20,000 cells/well) were washed with D-PBS and exposed to NM-pretreated or mock-treated virus at MOI of 1 at 33°C for 2 h. The virus inoculum was left in and cells were fed serum-free EMEM medium (100 μ l containing 1.0 μ g/ml TPCK-treated trypsin supplemented with various concentrations of NM or not, as indicated, in triplicate, and incubated further at 33°C. After 44 h, an equal volume of Takara lysing solution was added to each well and an aliquot of the total lysate was assayed for the level of influenza NP protein using the MK120 ELISA kit, as described under Vero cells. The data from these experiments in MDCK cells were plotted graphically to show the mean and SD of influenza A virus antigen level (units/ml) detected in lysates of triplicate cultures, as a function of NM concentration.

2.7. *Effect of NM and ascorbic acid on influenza A neuraminidase*

Titration of NA activity was performed by the method of Potier et al. [23] using cell-free influenza A virus, subtype H1N1 (strain A/WSN/33 from ATCC). The titer of undiluted virus stock used was $10^{5.75}$ TCID₅₀/0.2 ml in 3 days on MDCK cells at 33°C with 5% CO₂ by CPE. Kinetic analysis was first performed to determine optimal reaction time by incubating serial dilutions of virus in the presence of substrate solution, 1 mM 2'-(4-Methylumbelliferyl)- α -D-N-Acetylneuraminic Acid (MUN, prepared in 62.5 mM sodium acetate, pH 5.5) at 37°C for 90 mins. Reactions were stopped by addition of 150 μ l

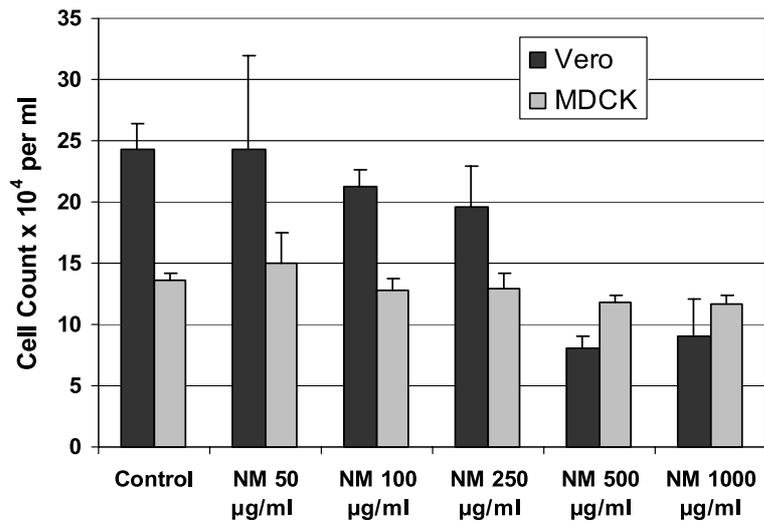


Fig. 1. Effect of NM on viability of MDCK and Vero cells. Cells were treated with various concentrations of NM and after 48 h, the viability was determined by the trypan blue dye exclusion test, as described in Methods and Materials. Shown is the viable cell count per ml as a function of NM concentration in Vero or MDCK cells.

of 0.1 M glycine buffer (pH 10.7) containing 25% ethanol. Absorbance was read with an Shimadzu spectrophotometer at 340 nm. From the above assay, 30 mins was chosen as the reaction time for further experiments. To investigate the effect of NM or AA on neuraminidase activity, cell-free virus was preincubated with various concentrations of test reagent for 0 or 60-minute at room temperature followed by assay of residual neuraminidase activity as above. For these experiments, stock solutions of the test agents were prepared in substrate buffer (62.5 mM sodium acetate, pH 5.5) and tested in the range of 50–3,000 µg/ml for NM and 100–3,000 µM for AA, in duplicate. Addition of AA or NM to the substrate buffer did not change the pH of the solution, which measured between 5.4–5.5.

2.8. Statistical analysis

The results for each representative study are expressed as means \pm standard deviation for the groups. Data were analyzed by independent sample 2-tailed *t* test. Pearson's correlation coefficients were determined for dose-dependent studies using MedCalc Software (Mariakerke, Belgium).

3. Results

3.1. Effect of NM on viability of MDCK and Vero cell lines

To provide a working concentration range for a therapeutic effect of NM, we initially evaluated the effect of various concentrations on the viability of Vero and MDCK cells. MDCK viability was not significantly affected after treatment with NM for 48 h, as assessed by the trypan blue dye exclusion test, as shown in Fig. 1. By this method, no toxicity was apparent at 50–250 µg/ml NM, with only slight lowering of viability (< 15% cell death) seen at 500–1000 µg/ml NM (correlation coefficient $r = -0.7632$, $p = 0.077$). Treatment of Vero cells with low concentrations of NM had little effect on cell viability, as measured by trypan blue exclusion method, producing 20% lowering of viable cell

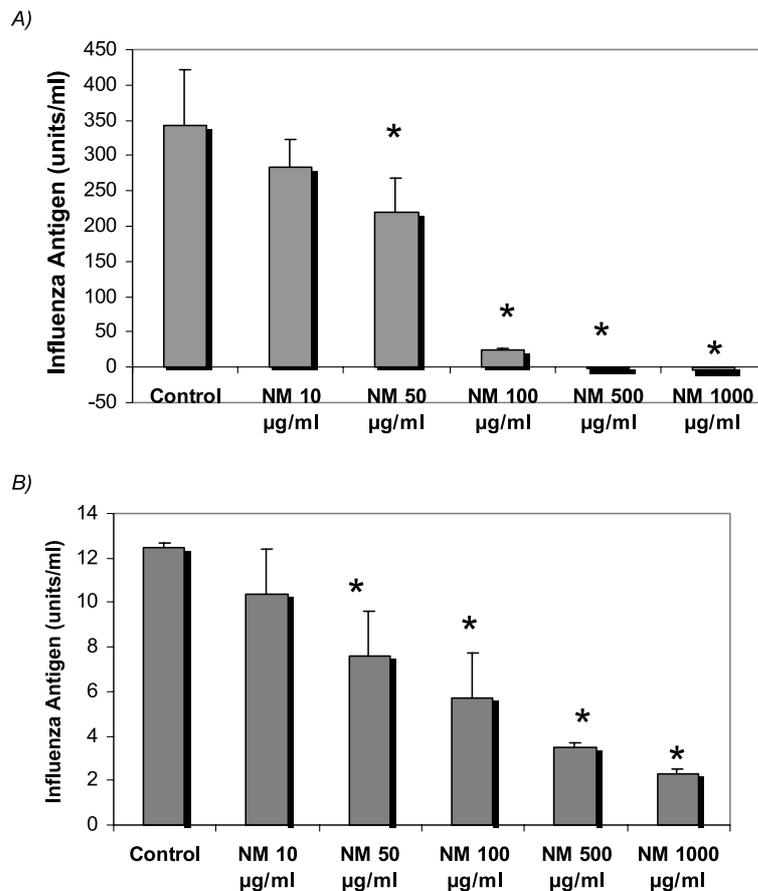


Fig. 2. Effect of NM on influenza total NP antigen production in Vero cells at 44–48 h. Cells (20,000 cells/well) were inoculated with 25 μ l/well influenza A virus strain A/WSN/33, MOI of 1 in the (A) presence or (B) absence of trypsin. Influenza antigen production was assayed as described in Methods and Materials, and is expressed in units per ml as a function of the NM concentration. (*) indicates significant difference from control by a two-tailed t-test.

count at 100–250 μ g/ml of the nutrient mixture (Fig. 1). At higher concentrations, 500–1000 μ g/ml NM, approximately 60–67% lowering in viability of Vero was observed (correlation coefficient $r = -0.8889$, $p = 0.018$). NM toxicity to cells is probably due to the effect of green tea extract on these cell lines, as reported by Song et al. [29]. Sensitivity of cells to NM varies among different cell lines, and thus explains the increased sensitivity to NM toxicity by Vero cells in contrast to MDCK cells. In regard to mechanisms, in a recent study (unpublished), we found that NM induced dose-dependent apoptosis in Vero cells, with approximately 69% of cells exhibiting late apoptosis at 500 μ g/ml NM, which appears to account for the cytotoxicity seen in Vero cells in the current study.

3.2. Effect of NM on influenza total NP antigen production in Vero cells

To delineate the antiviral effect of the nutrient mixture, Vero cells were infected with influenza A virus, exposed to various concentrations of NM and the titer of viral NP protein was determined as described in Methods. The results in Vero cells in the presence and absence of trypsin are shown in Fig. 2. Trypsin treatment increased influenza A NP antigen level from 12.5 units/ml (Fig. 2B) to 340 units/ml (Fig. 2A)

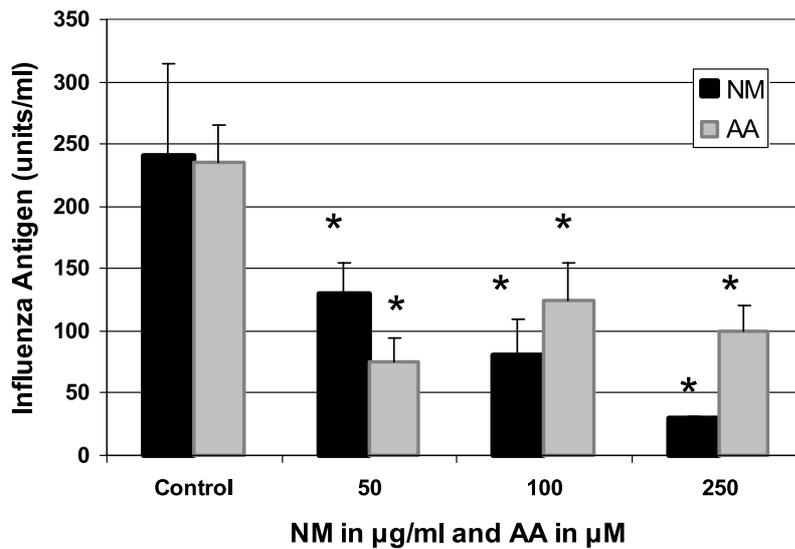


Fig. 3. Effect of NM and ascorbate (AA) on influenza total NP antigen production in Vero cells. Cells (50,000 cells/well) were inoculated with 50 μ l/well influenza A virus strain A/WSN/33, MOI of 1 in presence of trypsin. Influenza antigen production was assayed as described in Methods and Materials, and is shown as units per ml as a function of the NM or AA concentration. (*) indicates significant difference from control by a two-tailed t-test.

at 44–48 h after virus infection, consistent with the role of trypsin in culture as a prerequisite for viral infection. NP antigen production by influenza A virus in Vero cells treated with trypsin was inhibited profoundly by exposure to NM in a dose-dependent manner ($r = -0.7260$, $p = 0.102$) with 46% inhibition over control at 50 μ g/ml NM ($p = 0.088$), 93% at 100 μ g/ml NM ($p = 0.002$), and 100% at 500 and 1000 μ g/ml NM ($p = 0.001$), as shown in Fig. 2A. In the absence of trypsin, influenza A virus NP production in Vero cells was inhibited slightly less but in a dose-dependent manner ($r = -0.8221$, $p = 0.045$) with 40% inhibition over control at 50 μ g/ml NM ($p = 0.01$), 54% at 100 μ g/ml NM ($p = 0.004$), 72% at 500 μ g/ml ($p < 0.0001$) and 82% at 1000 μ g/ml NM ($p < 0.0001$), as shown in Fig. 2B.

Using a Vero culture exposed to influenza virus for a shorter period (24 h instead of 44 h) resulted in a slightly lower NP antigen level (240 vs. 340 antigen units/ml), as shown in Fig. 3. NM also inhibited influenza antigen NP production in a dose-dependent manner at 24 h, resulting in inhibition over control of 46% ($p = 0.070$), 67% ($p = 0.027$), and 87% at 50, 100, and 250 μ g/ml NM.

3.3. Effect of NM on influenza NP antigen production in MDCK cells and influence of cell or virus Pretreatment

The nutrient mixture also inhibited virus growth in a dose-dependent manner in MDCK cells (Fig. 4). The magnitude of inhibition was found to depend on conditions of cell and virus pretreatment. Pretreatment of MDCK cells with NM followed by washing of cells to remove NM then exposing to influenza virus inoculum (in the absence of NM) as described in Methods, resulted in a dose-dependent inhibition of NP antigen expression ($r = -0.8784$, $p = 0.1216$) with 38% ($p = 0.02$) reduction over control at 250 μ g/ml, 55% ($p = 0.0037$) at 500 μ g/ml and 62% ($p = 0.0023$) at 1000 μ g/ml (Fig. 4). Pretreatment of virus with NM, followed by infection and post-treatment of cells with NM (after exposure to virus), resulted in dose-dependent and more profound reduction of influenza NP protein expression ($r = -0.9385$, $p = 0.0615$) with 83% inhibition ($p = 0.004$) at 500 μ g/ml and 99% inhibition ($p = 0.003$)

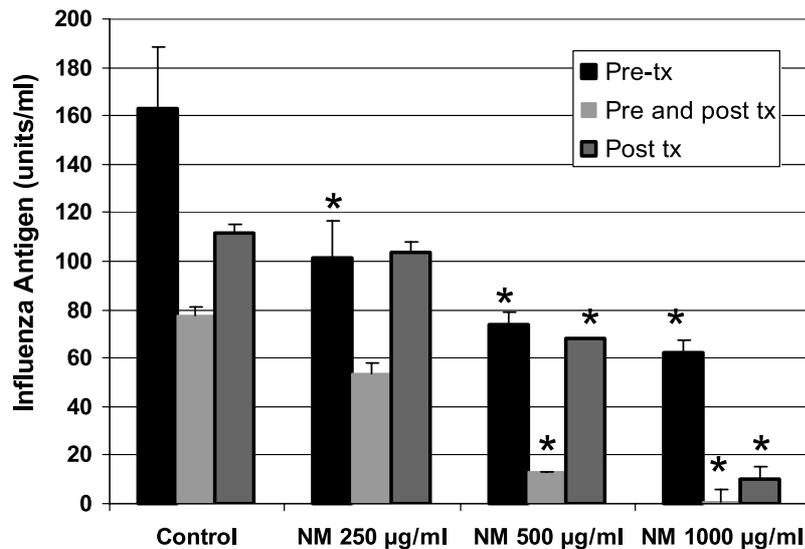


Fig. 4. Effect of NM on influenza NP protein expression in MDCK cells as a function of cell or virus pretreatment. Cells (20,000 cells/well) were inoculated with human influenza A virus strain A/WSN/33, at MOI of 1 in the presence of trypsin under three conditions: pretreatment of MDCK cells with NM prior to virus exposure; pretreatment of virus with NM prior to infection and treatment of cells with NM after exposure to virus; no pretreatment of cells with NM but addition of NM after exposure to virus as described in Methods and Materials. Shown is the influenza antigen level as a function of NM concentration under indicated conditions of treatment. (*) indicates significant difference from control by a two-tailed t-test.

at 1000 µg/ml (Fig. 4). No pretreatment of influenza virus or cells with NM, but adding NM to cells after virus infection resulted in more substantial inhibition than pretreatment alone but less than that observed with pre and post NM treatment ($r = -0.9850$, $p = 0.015$) with 39% inhibition ($p < 0.0001$) at 500 µg/ml and 91% inhibition ($p < 0.0001$) at 1000 µg/ml (Fig. 4).

3.4. Cytotoxicity and specificity of the antiviral effect of NM in vero and MDCK cells

To determine whether virus inhibition in Vero or MDCK cells is a specific effect of NM or due to the cytotoxicity of the mixture, we determined a selective index i.e. ratio of cytotoxicity value to antiviral efficacy value. Since trypan blue exclusion measures cell death but not cytostatic effects, we utilized a cytotoxicity assay based on measuring total biomass by staining cellular proteins with the dye Sulforhodamine B (Acid Red 52). This colorimetric method has been used successfully by Skehan et al. [28] to measure cytotoxic effects of anti-cancer compounds on cultured cells. We adapted this assay to determine the level of total protein in uninfected cells after treatment with various concentrations of NM as described in Methods. Antiviral activity was determined in parallel by assaying NP protein production in infected cells following treatment with corresponding concentrations of NM. Figure 5A shows the selective index (cytotoxicity value/antiviral value) in Vero cells and Fig. 5B shows the same index in MDCK cells after 48 h of NM treatment. In both cell lines, there was an improvement in the ratio of total cell protein to viral NP antigen with increasing concentrations of NM up to 200 µg/ml in Vero and 400 µg/ml in MDCK cells ($p = 0.047$ in Vero and 0.006 in MDCK at 200 µg/ml NM). These results are consistent with selectivity of the NM inhibitory effect towards influenza virus rather than due to inhibition of host cells.

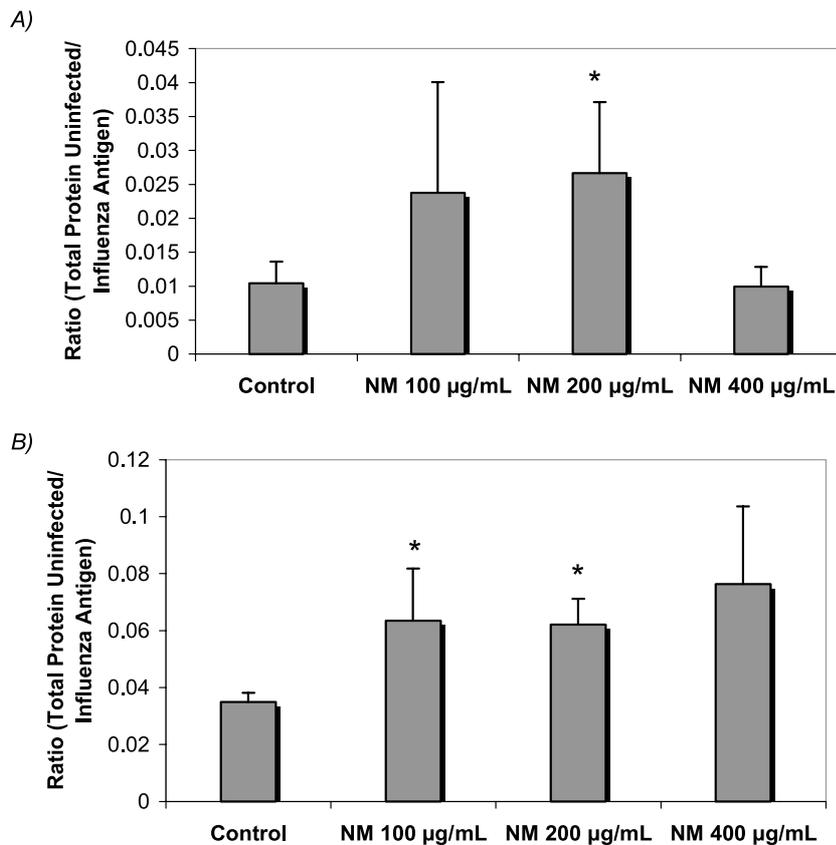


Fig. 5. Selectivity of antiviral effect of NM in Vero and MDCK cells. One-day old, sub-confluent monolayers of Vero cells (10,000 cells/well) or MDCK cells (20,000 cells/well) growing in 96-well plates were washed with PBS and exposed to human influenza virus (strain A/WSN/33 at MOI of 0.125) in serum-free EMEM medium containing 0.125% BSA (overlay, 25 μ l/well) or mock infected. The virus inoculation was left in and cells were fed serum-free overlay medium (100 micro liters containing 1.0 μ g/ml TPCK-treated trypsin) supplemented with various concentrations of nutrient mixture (NM) or no nutrient mixture as indicated above and incubated further at 33°C. After 48 h the supernatant was removed from each well, and cells were washed with PBS and then cells in each well were fixed and total protein was determined using the Sulforhodamine B based assay (Sigma). The supernatants collected were used to determine total influenza antigen (NP) units using the Influenza A Virus ELISA Kit (Takara). Ratios were calculated using total protein values and NP units obtained and are indicated in figure (A) for Vero cells and (B) for MDCK cells. (*) indicates significant difference from control by a two-tailed t-test.

3.5. Effect of individual NM component(s) on virus growth

One component of NM is green tea extract, which contains polyphenols that have been shown previously to inhibit influenza virus [22,29]. To determine whether additional components have antiviral activity, we evaluated ascorbic acid (AA, ascorbate or vitamin C), another constituent of NM. When tested on Vero cells, AA had some inhibitory effect on NP antigen production in trypsin-treated culture, with approximately 50% reduction at 100 and 250 μ M at 24 h (Fig. 3). Under the same conditions at a higher concentration range, AA also exerted dose-dependent suppression in MDCK cells (Fig. 6A). This action of ascorbate was virus specific as the selective index (total cell protein/viral NP antigen) increased with increasing vitamin concentration (Fig. 6B).

To determine whether individual components of NM display cooperative effects on virus inhibition, we tested ascorbic acid and green tea extract (GT), individually and in combination as shown in Fig. 7.

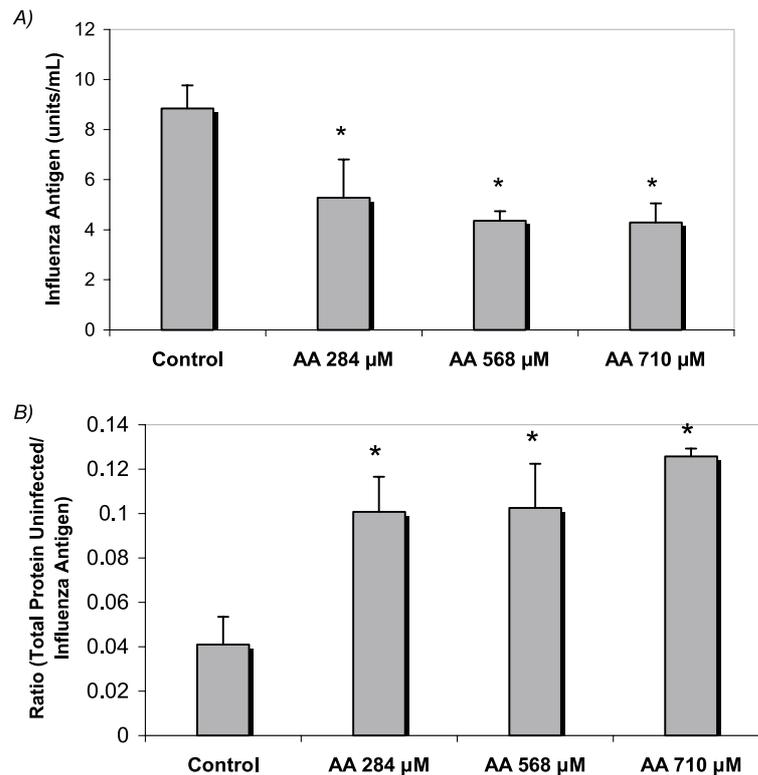


Fig. 6. Selective effect of ascorbic acid on NP production in MDCK cells. MDCK cells (20,000 cell/well) were infected, treated with indicated concentrations of ascorbic acid (AA) for 24 h and harvested essentially as described in Fig. 5 legend. The supernatants collected were used to determine total influenza antigen (NP) units using the Influenza A Virus ELISA Kit (Takara), and averages of quadruplicates are indicated in (A). Ratios were calculated using total protein values and NP units obtained and are indicated in (B). (*) indicates significant difference from control by a two-tailed t-test.

When evaluated individually at low to moderate concentrations, GT or AA produced slight inhibition of NP protein production in infected MDCK (Fig. 7A). When these components were combined, they conferred greater inhibition of viral NP protein than either component alone (Fig. 7A). From analysis of the data, the effect of the combination of AA and GT in inhibition of viral antigen appears to be synergistic rather than additive, as the magnitude of the effect of the combination is greater than the sum of the individual effects: 7.7% (GT alone) + 12.3% (AA alone) compared to 31.3% (GT and AA in combination). This effect was also selective as evidenced from the ratio of total cell protein to viral NP antigen (Fig. 7B).

3.6. Inhibitory effect of NM and ascorbic acid on influenza A virus neuraminidase activity in cell-free system

Since pretreatment of virus affected its growth in MDCK cells, we evaluated the effect of the nutrient mixture (NM) and ascorbic acid on neuraminidase activity of influenza A virus. For this purpose, the enzymatic assay of neuraminidase based on Potier et al. [23] was first adapted in our laboratory using cell-free virus as enzyme and MUN as substrate (see Methods). Neuraminidase activity was determined to be linear up to 90 minutes, with coefficient of correlation $r = 0.9931$ (data not shown). When cell-free

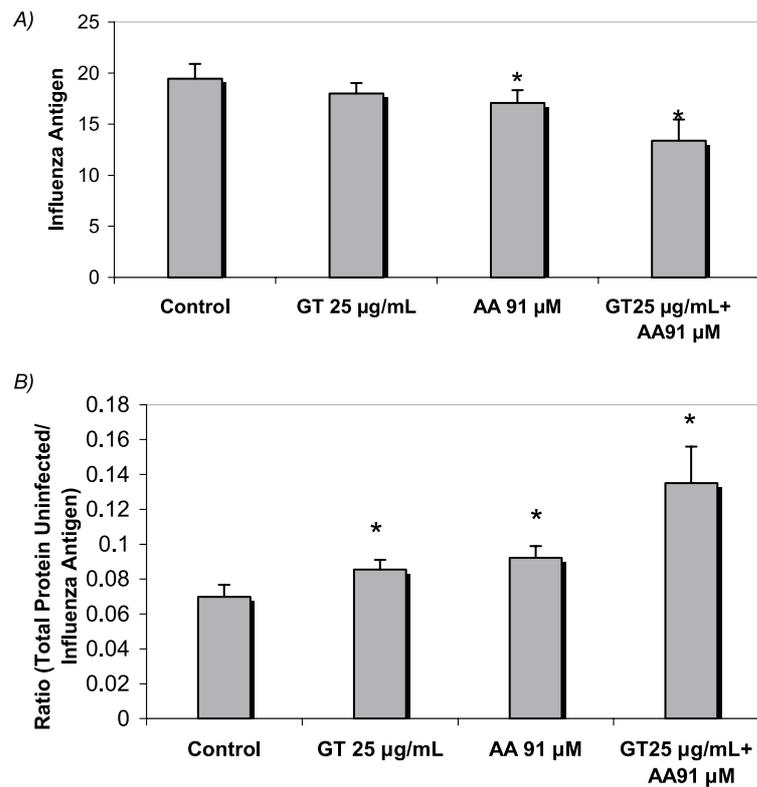


Fig. 7. Selective and cooperative effects of individual NM components in MDCK cells. MDCK cells (20,000 cells/well) were infected, treated with the individual components (individually or in combination) and after 72 h culture supernatants and cells were harvested and analyzed as described in Fig. 6. Averages of NP activity in quadruplicates are shown in (A) and ratio of total protein in uninfected cells to NP antigen units in infected cells in (B). (*) indicates significant difference from control by a two-tailed t-test.

virus was mixed with NM in the presence of substrate and the reaction stopped at 30 mins, the viral neuraminidase activity was inhibited in a dose-dependent manner ($r = -0.9221$, $p = 0.0089$), with 50% inhibition at 1000 $\mu\text{g/ml}$ NM, > 60% at 2000 $\mu\text{g/ml}$ NM, and 70% at 3000 $\mu\text{g/ml}$, as shown in Fig. 8. In contrast, neuraminidase activity was insignificantly inhibited (10–20% reduction) when NM was replaced with ascorbic acid (AA, 50–3000 μM), as shown in Fig. 8. The correlation coefficient r for ascorbic acid effect on neuraminidase was -0.5707 , $p = 0.2369$. Preincubation of cell-free virus with NM or AA for 60 minutes versus 0 minutes demonstrated similar neuraminidase activities (data not shown). In control experiments, addition of NM or AA to the assay tube (substrate buffer) did not change the pH of the buffer, suggesting that the effect on enzyme activity by the test reagent was not due to a pH effect.

4. Discussion

Therapeutic control of influenza virus infection can be directed at several targets. Among the known strategies for antiviral drugs are blocking viral uncoating through M2 ion channels (adamantanes) and inhibiting virus-associated neuraminidase activity linked to infectivity (Tamiflu, Relenza). However,

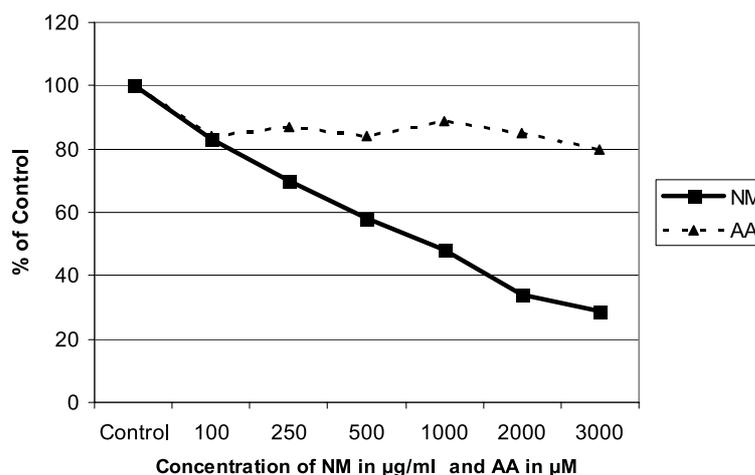


Fig. 8. Effect of NM and ascorbate (AA) on human influenza A neuraminidase activity. Cell-free virus was mixed with various concentrations of NM or AA and measurement of neuraminidase activity was performed in the presence of substrate as described in materials and methods. Shown is neuraminidase activity as percent of control at various concentrations of NM or AA.

these drugs have been associated with severe toxic side effects, suggesting the need for improved therapies. Another target is controlling viral growth through viral RNA, which contains all genes necessary for a virus to survive and reproduce in a host cell. The RNA is packed together with protein called NP (nuclear protein), which is needed by the virus to multiply its RNA copies [31]. In cells infected with a virus a decrease in NP protein production indicates that viral multiplication has been limited [31]. Our results demonstrated that the nutrient mixture (NM) containing lysine, proline, ascorbic acid, green tea extract, NAC, selenium and other micro nutrients is a potent inhibitor of influenza A growth (NP antigen production) as tested in MDCK and Vero cell cultures. This effect was not due to non-specific inhibition of viral NP resulting from NM-induced host-cell toxicity, since the selectivity index (ratio of cytotoxicity value to antiviral efficacy) increased with increasing concentration of NM indicating cell protection and selective inhibition of viral protein production. The antiviral effect was further enhanced upon pre-treatment of virus with nutrient mixture prior to infection and cell treatment with NM, suggesting the involvement of virus-associated component(s) as a possible target of action.

One surface component of influenza virus is the enzyme neuraminidase, which is linked to viral infectivity. By targeting the neuraminidase enzyme, the ability of viruses to escape from host cells and infect other cells can be hindered. Incubation of cell-free virus with NM showed dose-dependent inhibition of neuraminidase enzymatic activity. The inhibitory effect observed was not due to extraneous conditions such as pH change, since the pH of the substrate buffer in which NM was dissolved was in the 5.4–5.5 range, the optimum pH for the enzyme reaction [23].

Although NM inhibited neuraminidase activity, it is unlikely whether this is the main mechanism or the mode of inhibition of influenza virus by this mixture, since: (i) much higher concentrations of NM were required to inhibit NA activity than NP synthesis; (ii) NP inhibition was detectable within the first cycle of infection (at 24 hr), an unlikely occurrence if virion-associated neuraminidase was the sole target; and (iii) NM pre-treatment of virus alone without treatment of cells post-infection was less effective than pre plus post treatment. Other mechanisms are likely to be involved such as inhibition of a step(s) in viral replication. This is consistent with the inhibition of viral NP synthesis with individual components of NM and their known modes of action on other viruses and cancer cells.

A key component of NM is ascorbic acid, which decreased influenza A virus NP antigen production by over 50% at 50 μM –250 μM , but had negligible to slight effect only with increased dosage on influenza A neuraminidase activity. The pH of ascorbate solution (in culture medium) applied to cells was in the physiological range and that of the solution in substrate buffer used for the neuraminidase assay was also within the optimum range for the enzyme reaction, indicating that the above effects were not related to pH changes. Ascorbate was previously reported to inhibit HIV replication in lymphocytic cells [10], an effect that was localized to a post-transcriptional step in virus replication [11]. The vitamin was lowered in influenza virus-infected mice [14], which displayed increased oxidative stress in the lungs [3]. Ascorbate has been shown previously to selectively kill cancer cells through a pro-oxidant effect [5,18].

Another component of NM is green tea extract, which is enriched in polyphenols such as EGCG. Green tea extract has shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression [12]. Furthermore, EGCG and ECG were found to be potent inhibitors of influenza virus replication in MDCK cell culture studying various virus subtypes [15,29]. EGCG and ECG also suppressed viral RNA synthesis in MDCK cells (quantitative RT-PCR) and neuraminidase activity [29]. Total tea extract was more effective than any other isolated or mixture of polyphenols. In addition, EGCG agglutinated influenza virus in cell culture, preventing viruses from absorbing to MDCK cells [22]. We postulated that metabolic effects of a combination of nutrient components in NM would result from their synergy. In an earlier study, we reported that a combination of ascorbic acid, lysine and proline used with EGCG enhanced the anti-invasive activity of 20 $\mu\text{g}/\text{ml}$ EGCG to that of 50 $\mu\text{g}/\text{ml}$ [26]. In this study, we found that ascorbate and green tea extract cooperated to produce enhanced inhibition of influenza NP production (Fig. 8).

Other components in NM include amino acids (lysine, proline, arginine), amino-acid derivative (N-acetyl cysteine) and minerals (selenium, copper and zinc), which may also play a role in the suppression of influenza infection. These components were used to originally formulate the nutrient mixture (along with AA and green tea extract) based on targeting different physiological processes involved in cancer and infectious disease at the cellular level. For example, the extracellular matrix (ECM) integrity is dependent upon adequate collagen formation and its stability. Rath and Pauling suggested targeting plasmin-mediated mechanisms with the use of nutritional components, such as lysine and lysine analogues [24]. Collagen stability is supported by lysine [24] and also by N-acetyl cysteine through its inhibitory effect on MMP-9 activity [17] and invasive activities of tumor cells [19]. N-acetyl cysteine (NAC) was shown to significantly decrease mortality in influenza-infected mice [30]. Furthermore, in combination with ribavirin, NAC demonstrated a synergistic effect in protecting mice against a lethal influenza viral infection [9]. Manganese and copper are also essential cofactors in collagen formation process. Ghandi et al. [8] reported an antiviral effect directed towards influenza A virus by copper through inhibition of the proton translocation machinery in the M2 protein of this virus. Selenium has been shown to interfere with MMP expression and tumor invasion [33]. Selenium deficiency has been demonstrated to increase the pathogenicity of influenza virus infection in mice [2]. Increased oxidative stress from influenza virus, which results in increased NF- κB expression and enhanced TH-2 cell response and lung inflammation, was abated by adequate selenium intake, resulting in a TH-1 response after viral infection and reduced pathology. Further analysis of individual components of NM may provide more insight into the specific role(s) of these constituents in the inhibition of influenza virus multiplication.

Finally, in contrast to the toxic side effects of current influenza medications, the nutrient mixture has been shown to be a safe therapeutic agent with demonstrated utility. In a previous *in vivo* study addressing safety issues, we found that gavaging adult female ODS rats (weighing 250–300 gm) with the

nutrient mixture (at 30, 90 or 150 mg per day for seven days), had neither adverse effects on vital organs (heart, liver and kidney), nor on the associated functional serum enzymes, indicating that this mixture is safe to use even at these high doses, which far exceed the normal equivalent dosage of the nutrient, and can be delivered *in vivo* [25]. More recently, the nutrient mixture was shown to inhibit pathological effects in chickens resulting from avian flu [1].

In conclusion, the relatively non-toxic nutrient mixture tested in this report has potential in influenza treatment by selectively blocking viral multiplication and decreasing viral infectivity

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