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Inhibition of the infectivity of influenza virus by tea polyphenols

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Summary

(-)Epigallocatechin gallate (EGCg) and theaflavin digallate (TF3) (1–10 μM) inhibited the infectivity of both influenza A virus and influenza B virus in Madin-Darby canine kidney (MDCK) cells in vitro. Study by electron microscope revealed that EGCg and TF3 (1 mM) agglutinated influenza viruses as well as did antibody, and that they prevented the viruses from adsorbing to MDCK cells. EGCg and TF3 more weakly inhibited adsorption of the viruses to MDCK cells. EGCg and TF3 (1–16 μM) also inhibited haemagglutination by influenza viruses. These findings suggest that tea polyphenols bind to the haemagglutinin of influenza virus, inhibit its adsorption to MDCK cells, and thus block its infectivity.

Influenza virus; Tea; Polyphenol; Catechin; Theaflavin

Introduction

Tea extracts show bactericidal activity against various bacteria that cause diarrheal diseases (Toda et al., 1989a; 1989b; 1991) and inhibit the activity of their exotoxins (Okubo et al., 1989; Toda et al., 1991). Polyphenol is one of the components responsible for those activities of tea extracts (Ikigai et al., 1990; Toda et al., 1990).

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We recently found that tea extracts markedly inhibit the infectivity of both influenza A and B viruses for MDCK cells by blocking their adsorption to the cells (Nakayama et al., 1990). We therefore investigated the inhibitory activity of tea polyphenols against influenza viruses.

Materials and Methods

Tea polyphenols

(-)Epigallocatechin gallate (EGCg) and theaflavin digallate (TF3) were purified from green tea (Matsuzaki and Hara, 1985) and black tea (Hara et al., 1987), respectively, to purity exceeding 95% after separation by high-performance liquid chromatography, as described previously. Their molecular structures are shown in Fig. 1. They were dissolved in phosphate-buffered saline (PBS) at the appropriate concentrations before use.

Amantadine

Amantadine(1-aminoadamantane hydrochloride) was a gift of CIBA-GEIGY Japan (Hyogo, Japan). It was first dissolved in distilled water at 10 mM and then diluted with Eagle's minimum essential medium (MEM) before use.

Viruses

Influenza A/Yamagata/120/86(H1N1) and B/USSR/100/83 viruses were

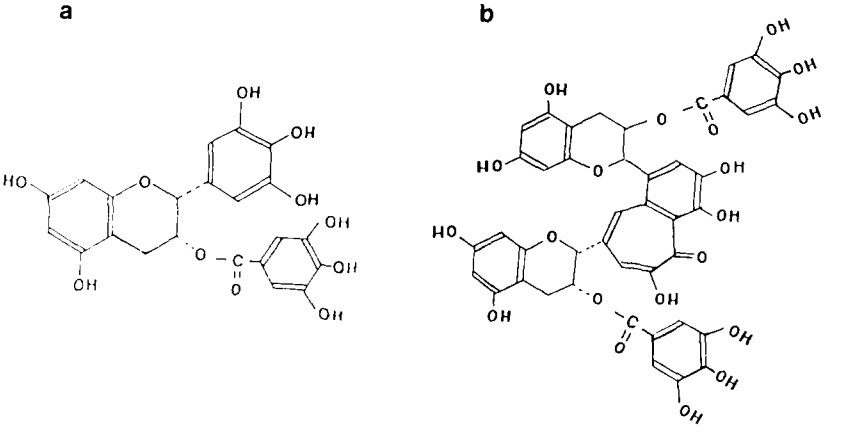

Fig. 1. Molecular structures of (-)epigallocatechin gallate (EGCg) (a) and theaflavin digallate (TF3) (b).

used. They were propagated in 11-day chick embryos and the infected allantoic fluids were stored at −80°C.

Cells

Madin-Darby canine kidney (MDCK) cells were maintained as described elsewhere (Tobita, 1975). MDCK cells were cultured as monolayers in MEM supplemented with 10% fetal calf serum (Flow, Australia).

Antibody

Japanese white rabbits weighing 3.0 kg were immunized i.c. and s.c. with the A viruses (32000 HA titer) in Freund's complete adjuvant two times with a 1 month interval. The sera were obtained after 2 weeks. IgG was prepared by affinity chromatography using protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden).

Plaque assay

Plaque assays were performed as described elsewhere (Tobita, 1975). Confluent monolayer MDCK cells in a 6-well tissue culture plate (Coster, MA, USA) were inoculated with a mixture of approximately 200 pfu viruses and polyphenol. After allowing 60 min for virus adsorption, the cells were washed twice with MEM, and overlaid with MEM containing 0.17% $NaHCO_3$, 200 μg/ml DEAE-dextran, 3 μg/ml trypsin, 0.9% noble agar, and antibiotics. After incubation for 4 days at 33.5°C in 5% CO_2 in air, the cells were fixed with 10% formalin for 30 min, and the agar overlay was then removed by flooding with tap water. The attached cells were stained with 0.038% methylene blue, the plaques were counted and the percentage of plaque inhibition was calculated.

Cytotoxicity assay

Polyphenol at various concentrations was added to MDCK cells (5.7×10^5 cells/ml), and the cells were incubated for 40 h. After incubation, the cells were harvested and viable cells were counted by nigrosine staining. The percentage of cell viability was then calculated.

Treatment of virus with polyphenol or antibody for electron microscopy

The A viruses (32000 HA titer) purified by sucrose density gradient centrifugation were incubated with 1 mM polyhenol or antibody (6400 HI titer) for 5 min or 60 min at 37°C.

Electron microscopy

Negative staining. The A viruses mixed with polyphenol or antibody for 5 min were adsorbed to carbon-coated collodion films on copper grids. The viruses on the grids were stained with 2% sodium phosphotungstate (pH 7.2) (Brenner and Horne, 1959) and examined under an Hitachi H-7000 electron microscope at 75 KV.

Scanning electron microscopy. The A viruses mixed with polyphenol or antibody for 60 min were laid on MDCK cells grown on the cover slips for 1 min at room temperature. The treated MDCK cells were washed 3 times with PBS, prefixed with 2.5% glutaraldehyde-2.0% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 60 min at room temperature following two washes with PBS, and were post-fixed with 2% OsO_4 in the same buffer for 45 min. After thorough washing, the cells were dehydrated in ethanol, treated with isoamyl acetate, and dried to a critical point with HCP-2 (Hitachi, Tokyo, Japan). The cells were mounted on stubs, coated with gold at 3.5 cm with 2–2.5 KV and 7 mA for 4 min (Ion coater IB-5, RMC-EIKO, Kawasaki, Japan) and observed with an Hitachi S-700 scanning electron microscope.

Haemagglutination assay

25 μl of A virus suspension were mixed with an equal volume of polyphenol and maintained for 5 or 60 min at room temperature. Each of these mixtures was then diluted 5 times, 2-fold dilution each time. 50 μl of the original solution and all dilutions of the mixture were then incubated with an equal volume of 0.5% chicken erythrocyte (CRBC) suspension for 60 min at room temperature for haemagglutination.

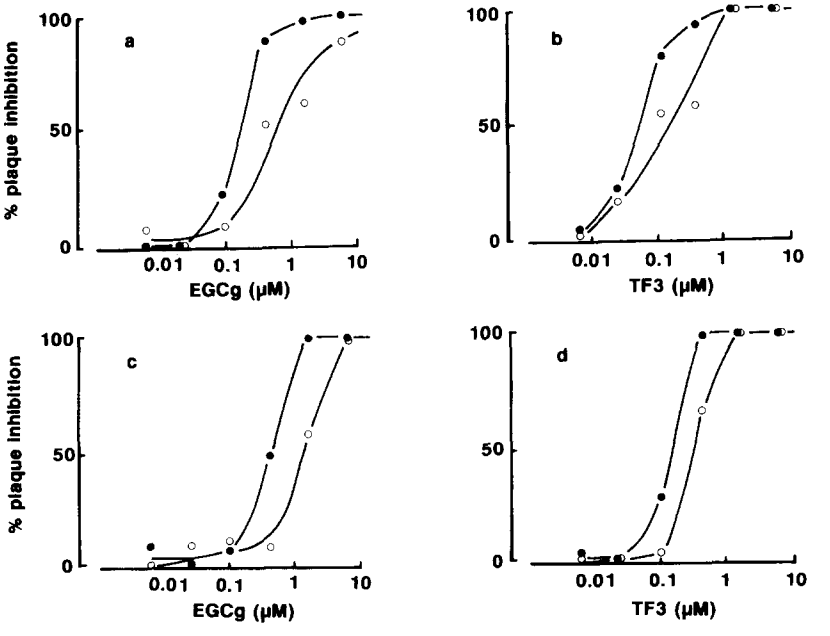

Fig. 2. Inhibitory effects of EGCg and TF3 on plaque formation induced by influenza A virus (a,b) and B virus (c,d). Influenza virus stocks were diluted to 2×10^3 p.f.u. ml^{-1} and incubated with various concentrations of EGCg (a,c) or TF3 (b,d) for 5 min (○) or 60 min (●) at 37°C before virus exposure to MDCK cells. The inhibition of plaque count was scored by the mean of triplicate cultures for each group after assay. Mean p.f.u. ± S.D. was 78.8 ± 22.1 of control of eight experiments.

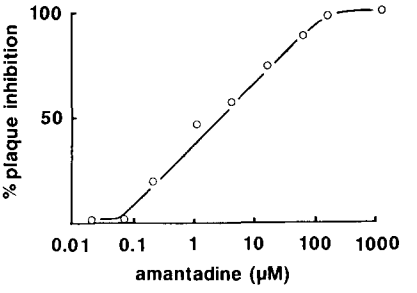

Fig. 3. Inhibition of plaque formation by amantadine. Influenza A virus was incubated with various concentrations of amantadine for 10 min at 37°C before virus exposure to MDCK cells. The inhibition of plaque count was scored by the mean of triplicate cultures for each group after assay. Mean p.f.u. ± S.D. was 170.3 ± 14.2 of control ($n = 9$).

Results

Inhibition of plaque formation by polyphenol

We first investigated the capacity of EGCg and TF3 to inhibit infection of influenza A and B viruses in MDCK cells. The virus was mixed with EGCg or TF3 for either 5 min or 60 min before being exposed to the cells. EGCg and TF3 inhibited the infectivity of both viruses (Fig. 2). Even concentrations as low as 1.5 μM ECCg or TF3 inhibited almost 100% of the plaque forming activity of the viruses after 60 min treatment. Short-time contact (5 min) of EGCg or TF3 with the viruses also effectively inhibited the infectivity. Amantadine was used as a control. Influenza A virus was mixed with amantadine for 10 min before being exposed to MDCK cells. The concentration of amantadine required to inhibit plaque formation by influenza A virus was approximately 50–100 times that of polyphenols (Fig. 3).

We further determined whether polyphenols are effective if added after adsorption of virus to MDCK cells. Influenza A viruses were exposed to the

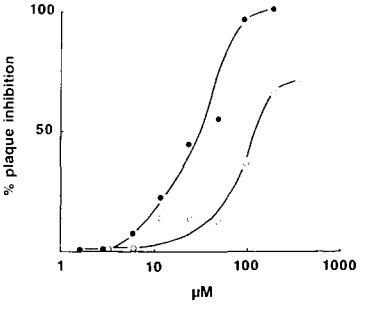

Fig. 4. Inhibitory effects of EGCg and TF3 on viruses adsorbed to MDCK cells. Influenza A virus was exposed to MDCK cells for 30 min at 4°C. EGCg (○) or TF3 (●) at various concentrations was then added to virus-adsorbed cells for 15 min and the cells were washed twice with MEM and cultured. The inhibition of plaque count was scored by the mean of quadplicate cultures for each group after assay. Mean p.f.u. ± S.D. was 88.8 ± 10.0 of control ($n = 12$).

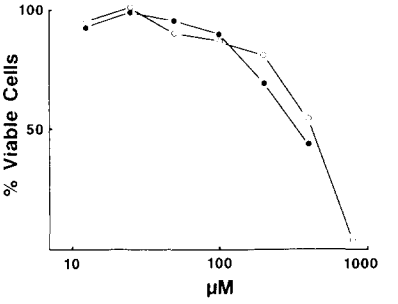

Fig. 5. Toxicity of EGCg and TF3 to MDCK cells. MDCK cells were incubated in the presence of EGCg (○) or TF3 (●) for 40 h as described in Materials and Methods. Viable cells in duplicate cultures were counted and the percentage of cell viability was calculated. Mean viable cells was 1.02×10^6/ml of control ($n = 3$).

cells at 4°C for 30 min. Polyphenol was then added to virus-adsorbed cells for 15 min and the cells were washed twice with MEM and cultured. Although the effective concentration of polyphenol was higher, inhibition of plaque formation did occur (Fig. 4). However, when polyphenol was added 30 min or more after adsorption of the A virus to MDCK cells at 37°C, plaque forming activity of the virus was not inhibited by polyphenols at any concentration (data not shown).

Furthermore, when MDCK cells were pretreated with polyphenols and washed to remove residual polyphenols, and then challenged with the virus, plaque formation was not inhibited at concentrations of 100 μM or less (data not shown).

EGCg and TF3 concentrations greater than 200 μM and 100 μM, respectively, were toxic to MDCK cells (Fig. 5).

Observation by electron microscopy

The above results suggest that tea polyphenols may bind to surface glycoproteins of the influenza virus. We investigated this possibility by electron microscopy. We compared the capacities of EGCg, TF3 and anti-A virus (H1N1) antibody to bind to the A virus. EGCg and TF3 (1 mM) agglutinated virus particles as well as the antibody during short-time contact (Fig. 6). Viruses pretreated with EGCg (1 mM), or, with the antibody failed to bind to MDCK cells (Fig. 7).

Inhibition of haemagglutination by polyphenol

Observation by electron microscopy suggests that tea polyphenol binds to HA antigens and prevents virus adsorption to cells. We finally investigated the capacity of EGCg and TF3 to inhibit haemagglutination induced by influenza virus. Like antibody, EGCg and TF3 (1–16 μM) inhibited haemagglutination (Fig. 8).

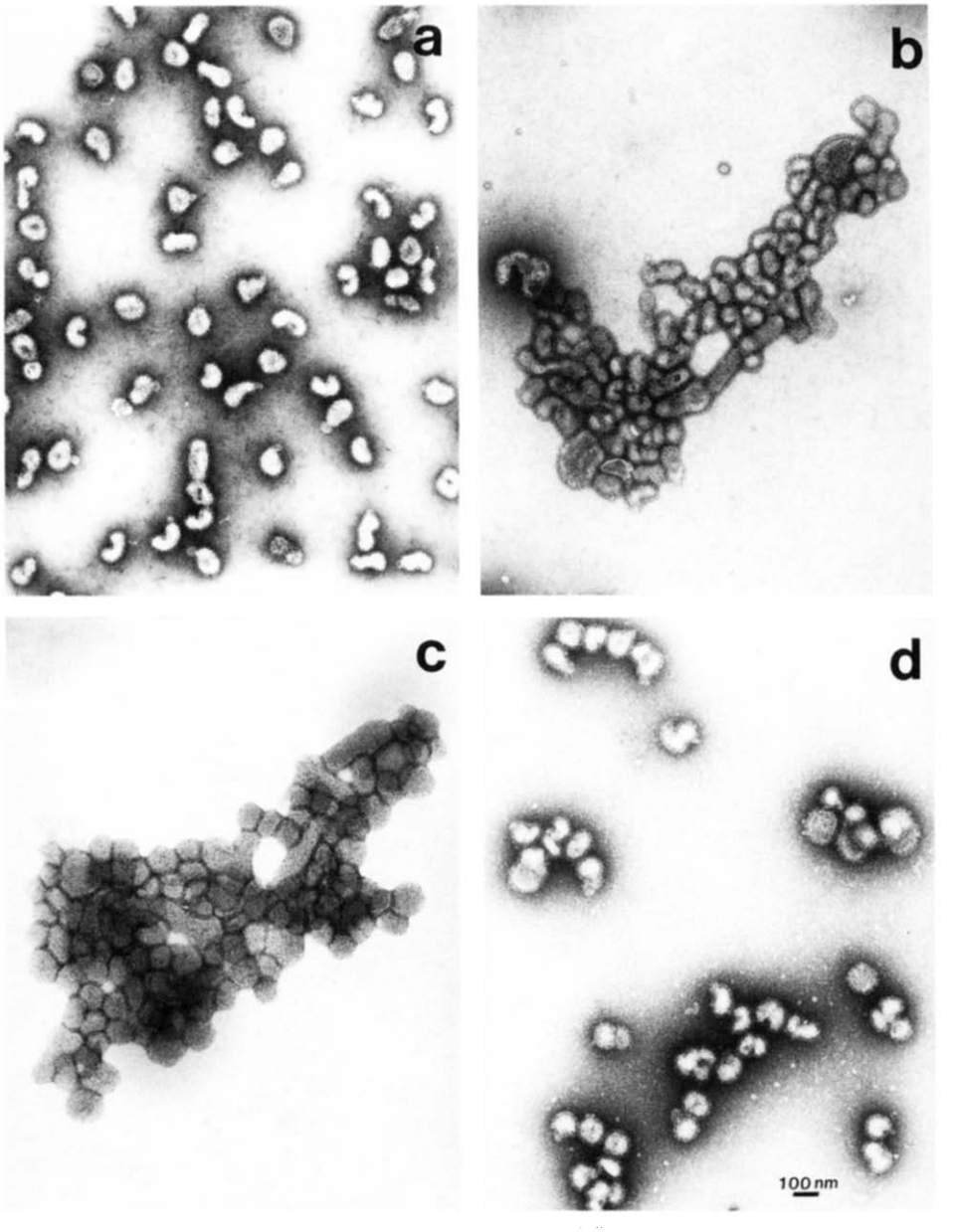

Fig. 6. Electron micrographs of influenza A virus after incubation for 5 min with PBS as control (a), 1 mM EGCg (b), 1 mM TF3 (c) or anti-A virus IgG (6400 HI titer) (d). Viruses were negatively stained with 2% sodium phosphotungstate (pH 7.2) for electron microscopy.

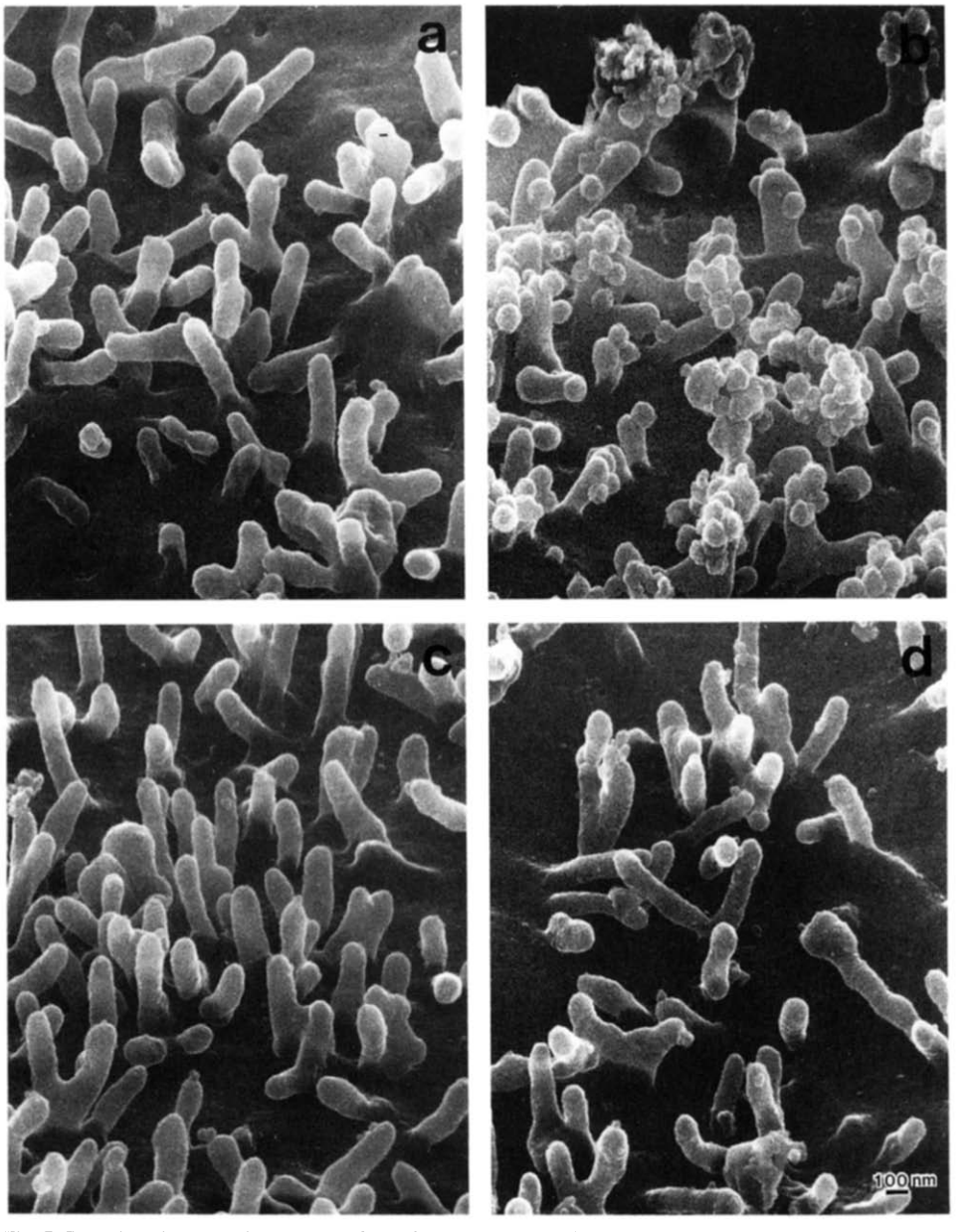

Fig. 7. Scanning electron micrographs of MDCK cells after incubation with influenza A virus pretreated by PBS (b), 1 mM EGCg (c) or anti-A virus IgG (6400 HI titer) (d). MDCK cells without the A virus as control (a).

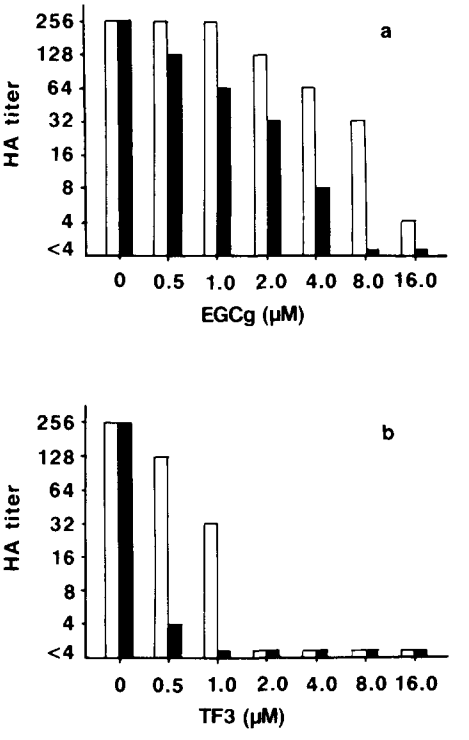

Fig. 8. HA titers of influenza A virus treated with various concentrations of EGCg (a) or TF3 (b) for 5 min (open column) or 60 min (solid column).

Discussion

Influenza is a disease with a high mortality rate throughout the world. Despite efforts to develop effective vaccines and therapeutic agents against influenza virus infection, it is still virtually uncontrolled. The use of presently developed vaccines against influenza virus infection is limited because of the frequent conversion of viral antigens. Symptomatic therapy is the only treatment possible for influenza virus infection except in a few countries where the antiviral compound, amantadine, is used.

Although amantadine is effective in prophylaxis and therapy of influenza A virus, it has side effects, and resistant mutants arise (Dolin, 1985). Therefore, new vaccines and antiviral strategies are being explored (Wilson and Cox, 1990). Our results might provide new strategies against influenza virus infection.

The results indicate that tea polyphenols can inhibit the infectivity of influenza virus to MDCK cells by blocking its adsorption and entry into the cells, but not its multiplication inside the cells. We previously reported that tea extract inhibited the infectivity of both influenza A and B viruses by a similar mechanism (Nakayama et al., 1990). Beverage concentrations of tea contain at

least 500 μM EGCg. Taken together, it appears that polyphenols are responsible for the anti-influenza virus activity of tea extract. Green (1949) reported that black tea extract inhibited the multiplication of influenza A virus in embryonated eggs. The discrepancy between the mechanism of action against influenza virus in our results and those of Green may be due to the experimental systems.

The precise molecular mechanism of antiviral activity of tea polyphenols is unknown at present. Tea polyphenols, unlike antibody, are nonspecifically effective against both influenza A and B viruses. They did, however, show HA antigen binding properties similar to that of antibody. They inhibited the infectivity of influenza virus at 0.0025–0.005 times the concentration that has a side effect on MDCK cells. The results reported here are restricted to cells in vitro. Therefore, further investigation is necessary to confirm that tea polyphenols are useful as prophylactic agents against influenza virus infection.

Acknowledgements

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