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Short Communication

**INHIBITION OF ROTAVIRUS AND ENTEROVIRUS INFECTIONS
BY TEA EXTRACTS**

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SUMMARY: Epigallocatechin gallate from green tea and theaflavin digallate from black tea inhibited infections of cultured rhesus monkey kidney MA 104 cells with rotaviruses and enteroviruses. Their antiviral effects were maximally induced when directly added to virus, and their pre- and post-treatment of the cells produced much weak antiviral activity. Antiviral activity of the extracts therefore seems to be attributable to interference with virus adsorption.

There is a Japanese folk legend telling that oral administration of tea is an effective medicine for acute gastroenteritis in children. Tea leaves have been reported to have bactericidal activity against *Vibrio cholerae*, *Salmonella typhi* and *Staphylococcus aureus* which cause diarrheal diseases (1) and anti-viral activity against influenza virus (2), vaccinia virus, herpes simplex virus,

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Coxsackie virus B6 and poliovirus 1 (3). Green tea extracts, namely epigallocatechin gallate (EGCg) and epicatechin gallate, inhibit infection of a cell culture with a strain of rotavirus (4). We attempted to examine tea extracts for the effects on enteric viruses in cell cultures.

Materials: EGCg: 100 g of commercial hot water-extracted green tea (Nakai-Hoto En Ltd. Co., Ujiyamada, Japan) was put into 1,000ml of water and an equal volume of chloroform was added. After shaken vigorously, the aqueous layer was mixed with three volumes of ethyl acetate. The ethyl acetate layer was evaporated and freeze-dried. The extracted crude catechin mixture, approximately 30 g, was fractioned by high performance liquid chromatography (Waters Prep LC/System 500 A, Millipore Co., MA). Epigallocatechin gallate (EGCg) (12 g), epigallocatechin (EGC) (3.5 g), epicatechin gallate (ECg) (3.0 g) and epicatechin (EC) (2.0 g) were separated (5). TF-3: 10 g of commercial hot water-extracted black tea (Lipton Tea, Lipton Ltd. Co., London) was put into 500 ml of water and an equal volume of chloroform was added. The aqueous layer was extracted with an equal volume of methyl-iso-butyl ketone (MIBK). The MIBK layer was washed with an equal volume of 2.5% NaHCO₃, evaporated and freeze-dried. This crude theaflavin, 300 mg, was fractioned by high performance liquid chromatography (Shimadzu LC4A, Shimadzu Co., Kyoto). Free theaflavin (TF-1), theaflavin monogallate (TF-2) and theaflavin digallate (TF-3) were obtained (6) (Fig. 1).

Epigallocatechin gallate (EGCg)

Theaflavin (TF-3)

Fig. 1. Structures of epigallocatechin gallate (EGCg) and theaflavin digallate (TF-3).

Viruses and cells: Human rotavirus (HRV) strains Wa (serotype 1), TMC2 (serotype 2), YO (serotype 3), Hocht (serotype 4) and 69 M (serotype 8) were kindly supplied by Drs. Inaba and Ikegami. Poliovirus 1 strain Sabin 1, Coxsackie virus A16 strain G-10, Coxsackie virus B3 strain Nancy and ECHO virus 11 strain Gregory were the stocks of National Institute of Health, Tokyo. These viruses were cultured and adapted in rhesus monkey kidney cell line MA 104 cells (7). Infectivities of rotaviruses and enteroviruses were determined by cytopathic effect (CPE).

Assay for anti-rotavirus and -enterovirus activities: Cells were grown as a monolayer culture in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal calf serum in 96-well tissue culture plates. Virus suspensions diluted to a concentration of 200 TCID₅₀ in 96-well microplates were mixed with serially diluted EGCg or TF-3 for 1 hr and then added to each well. After adsorption for 30 min, the wells were washed twice with DMEM, and then cultured for 7 days with DMEM. The cultures were observed under a microscope for CPE every day for a week.

Five wells were used for each concentration of a sample. The results were shown as percent inhibition of CPE calculated by the following formula at each concentration of a sample: $100 (\%) \times [5 - \text{numbers of wells with positive CPE}] / 5$.

Effects of EGCg on rotaviruses and enteroviruses: Figure 2-A shows that 50% inhibition of CPE by EGCg occurred at about 8 $\mu\text{g/ml}$ for Wa and 69 M, at about 32 $\mu\text{g/ml}$ for TMC2 and Hocht, and at about 62.5 $\mu\text{g/ml}$ for YO; 100% inhibition of CPE was noted at a concentration of 125 $\mu\text{g/ml}$. Cytotoxic effect of EGCg as shown by the trypan blue dye exclusion test appeared at concentrations higher than 1,000 $\mu\text{g/ml}$. Figure 2-B shows the effect of EGCg on enterovirus infections. At about 15 $\mu\text{g/ml}$ for Sabin 1 and about 125 $\mu\text{g/ml}$ for G-10, Nancy and Gregory occurred 50% inhibition of CPE; 100% inhibition of CPE was noted at 250 $\mu\text{g/ml}$ for all enteroviruses examined.

Effects of TF-3 on rotaviruses and enteroviruses: Figure 2-C shows that 50% inhibition of TF-3 occurred at about 15 $\mu\text{g/ml}$ for Sabin 1, at about 32 $\mu\text{g/ml}$ for Wa and, at about 62.5 $\mu\text{g/ml}$ for TMC2 and Nancy; 100% inhibition of CPE was noted at 250 $\mu\text{g/ml}$ for all viruses examined. The cytotoxic effect of TF-3 became apparent at concentrations higher than 1,000 $\mu\text{g/ml}$.

Effects of pretreatment with EGCg: 250 $\mu\text{g/ml}$ of EGCg in DMEM was added to cells 48, 24 and 12 hr before virus inoculation. After washing, Wa, TMC2, and Sabin 1 were adsorbed at 1,000 TCID₅₀ per well. Twelve, 24, and 48 hr after inoculation, culture fluids and cells were collected separately. The three viruses

Fig. 2. Inhibition of rotavirus and enterovirus infection by EGCg and TF-2. A: Effect of EGCg on (○) Wa; (●) TMC2; (□) YO; (■) Hocho; (△) 69M. B: Effect of EGCg on (○) Sabin 1; (●) G-10; (□) Nancy; (■) Gregory. C: Effect of TF-3 on (○) Wa; (●) TMC2; (□) Sabin 1; (■) Nancy.

were not detected by the CPE method as described above in the supernatant fluids nor in the cell fractions at 12 hr after inoculation. On the other hand, the viruses were detected in 48 hr after inoculation in cultures pretreated with EGCg for 24 and 48 hr. The viruses grew faster in untreated cells than in EGCg-treated cells (data not shown).

Table I. Effect of post-treatment with epigallocatechin gallate

Harvest (hr)	Virus	Virus replication			
		EGCg(+)		EGCg(–)	
		Sup.	Cell Fr.	Sup.	Cell Fr.
12	HRV-Wa	–	–	–	+
	HRV-TMC2	–	–	–	+
	Sabin 1	–	–	+	+
24	HRV-Wa	–	–	+	+
	HRV-TMC2	–	–	+	+
	Sabin 1	–	–	+	+
48	HRV-Wa	–	–	+	+
	HRV-TMC2	+	+	+	+
	Sabin 1	+	+	+	+
96	HRV-Wa	+	+	+	+
	HRV-TMC2	+	+	+	+
	Sabin 1	+	+	+	+

Infectivity was determined by CPE. + and – mean positive and negative CPE, respectively. Sup and Fr means supernatant and fraction, respectively.

Effects of post-treatment with EGCg: Wa, TMC-2 and Sabin 1 at 1,000 TCID₅₀ were inoculated to the cells, which were incubated for 1 hr. After washing, 250 µg/ml of EGCg in DMEM was added. Twelve, 24, 48 and 96 hr later, the culture fluids and cells were collected separately and the infectivities were examined by CPE. In the culture fluids and cells at 12 in 24 hr after inoculation, the viruses were not detected. The effect of post-treatment with EGCg were transient, since the viruses were detected in the supernatant fluid and cells in 48 and 96 hr after inoculation (Table I).

Rotavirus is an important agent causing gastroenteritis especially in children. Poliovirus, ECHO virus 11, Coxsackie virus A 16 and Coxsackie virus B3 cause poliomyelitis, meningitis, hand-foot-mouth disease and carditis, respectively. We examined the effectiveness of EGCg from green tea and TF-3 from black tea on different strains or groups of enteric viruses. Poliovirus 1 and two strains of rotaviruses (Wa and 69M) were more sensitive to EGCg than Coxsackie virus A16, Coxsackie virus B3 and ECHO virus 11. TF-3 was also

effective on these viruses. We do not know now why the effectiveness differed from one virus to another.

Growths of rotavirus and poliovirus were inhibited most effectively when EGCg was added to cells simultaneously with virus and less effectively by pre- and post-treatments with EGCg. No explanation can be given why pre-treatment is not so effective. One possible reason is the inactivation of EGCg when kept in culture medium for more than a day: the color of EGCg in the solution changes to brown or dark. The antiviral activity of EGCg is probably attributable to the interference with the virus absorption. It is, however, needed to examine the possibility that EGCg enters the cells and thereby inhibits the virus replication.

One to 2 mg/ml of EGCg is contained in green tea which we drink daily. Our experiments indicate that EGCg and TF-3 might be preventive and therapeutic agents against enteric virus infections.

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