CURRENT MICROBIOLOGY Vol. 47 (2003), pp. 244–249
DOI: 10.1007/s00284-002-3956-6

Current Microbiology
An International Journal

A Combination Effect of Epigallocatechin Gallate, a Major Compound of Green Tea Catechins, with Antibiotics on *Helicobacter pylori* Growth In Vitro

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Received: 2 September 2002 / Accepted: 12 November 2002

Abstract. Since green tea catechins are known to have antimicrobial activity against a variety of microorganisms, their possible effects on *Helicobacter pylori* in combination with antibiotics were examined. Fifty-six clinical isolates of *H. pylori*, including 19 isolates highly resistant to metronidazole (MTZ) and/or clarithromycin (CLR), were used to determine in vitro sensitivity to tea catechins. The MIC_{90} of both epigallocatechin gallate (EGCg) and epicatechin gallate (ECg) was 100 μg/ml. However, other tea catechins tested did not show any anti-*H. pylori* activity. Highly antibiotic-resistant clinical isolates showed a similar sensitivity to both EGCg and ECg. The kinetic study of antibacterial activity in liquid cultures revealed a relatively slow but strong activity on the growth of *H. pylori*. In combination with sub-MIC of amoxicillin (AMX), the antibacterial activity of AMX was significantly enhanced by the presence of EGCg. To estimate the general combination effect between EGCg and other antibiotics, such as MTZ and CLR, on the antibacterial activity against clinical isolates, the fraction inhibitory concentration (FIC) was determined by checkerboard study. The FIC indexes showed additive effects between EGCg and antibiotics tested. These results indicate that EGCg may be a valuable therapeutic agent against *H. pylori* infection.

Tea is the second most common beverage consumed by humans. Although this beverage has little nutritional value per se, tea is refreshing, mildly stimulating, and produces a feeling of well-being. Current extensive studies indicate that tea has some beneficial health effects besides being refreshing. The antitumor as well as antimicrobial activity of tea extracts and tea components have some beneficial health effects demonstrated by in vitro studies [1, 10, 13]. The active components of tea responsible for such biological activities are now recognized to be catechins (also known as polyphenols), which constitute catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), epicatechin gallate (ECg), and epigallocatechin gallate (EGCg). Among these tea catechins, EGCg is a major form and the most active in regard to a variety of biological activities.

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Tea extracts, including EGCg, have already shown a strong antimicrobial activity at "cup-of-tea" concentrations against a wide variety of microorganisms, such as methicillin resistant *Staphylococcus aureus* (MRSA), many enteropathogenic bacteria, and other microorganisms, even fungi [22–24]. However, the antimicrobial activity of tea is not extended to all microorganisms. For example, tea is active against *Mycoplasma pneumoniae* and *M. orale*, but not against *M. salivarium* [5]. Some yeasts, such as *Candida albicans* and *Cryptococcus neoformans*, are also resistant to the antimicrobial activity of tea, even though *Trichophyton mentagrophytes* and *T. rubrum* are susceptible [22]. A study of the mechanism of the antibacterial activity of tea catechins showed that the primary target site of catechins is phospholipids of bacterial membrane [11]. Therefore, even though much is not clear about antibacterial mechanisms, bacterial

membrane perturbation may be at least one of the reasons why catechins show strong direct antibacterial activity [7].

Helicobacter pylori is known to be an etiological agent of chronic gastritis and peptic ulcer as well as a risk factor for development of gastric cancer [3, 6, 18]. Eradication of *H. pylori* by antibiotics is thought to be an essential therapy for such gastric diseases. In this regard, current triple therapy with antibiotics and a proton pump inhibitor shows a high eradication rate and a low incidence of harmful side effects [2, 21, 26]. Although high eradication success in clinical trials has been reported, some problems still remain for this therapy, such as non-compliance and occurrence of resistance to antibiotics [16, 20]. Therefore, exploration of the possibility of using other therapeutic as well as prophylactic agents has been proposed. In this regard, current studies in our laboratories and others [17, 29] indicate that tea catechins might be a possible alternative therapeutic agent for gastric diseases caused by *H. pylori* due to its physicochemical stability in stomach acid [8], nonantibiotic nature, and safety. However, the detail of anti-*H. pylori* activity of catechins, particularly against antibiotic-resistant bacteria known to cause failure of eradication by antibiotics, as well as modulatory effects on the antibacterial activity of antibiotics utilized for patients as an eradication therapy for *H. pylori* infection, is not known. In the present study, therefore, in order to examine the detail of anti-*H. pylori* activity of tea catechins, the antibacterial activity against antibiotic-resistant *H. pylori*, and the effect of catechins on bacterial growth in combination with antibiotics were examined.

Materials and Methods

Bacterial strains. Fifty-six clinical isolates of *H. pylori*, including 19 isolates highly resistant to metronidazole (MTZ) and/or clarithromycin (CLR), isolated from patients undergoing upper gastrointestinal examination were kindly provided by K. Tamura, Hyogo College of Medicine, Nishinomiya, Japan, and Takeda Chemical Industries, Ltd., Osaka, Japan. A reference strain of *H. pylori* (ATCC43504) was provided by A. Nakazawa, Yamaguchi University School of Medicine, Yamaguchi, Japan. The bacteria were cultured on Bacto brucella agar (Difco Laboratories, Detroit, MI) supplemented with 7% horse defibrinized blood for 3–4 d at 37°C in a microaerobic atmosphere generated by a Pack-Campylo (Mitsubishi Gas Chemical, Tokyo, Japan).

Catechins and antibiotics. Catechins provided by Mitsui Norin Co., Fujieda, Japan were dissolved in water, sterilized with a membrane filter and then diluted with sterilized water. MTZ and amoxicillin (AMX) were purchased from Sigma, St. Louis, MO and dissolved in water. CLR provided by Dainabot Co., Osaka, Japan was dissolved in ethanol.

MIC determination. MICs were determined by the agar dilution method. Each catechin or antibiotic was incorporated into brucella agar supplemented with 7% horse blood. Plates contained twofold serial dilutions of catechins from 6.25 to 200 μg/ml or 0.013 to 100 μg/ml antibiotics. A suspension of approximately 10^9 bacteria/ml was prepared in brucella broth medium using a 72-h culture of *H. pylori* on 7% blood-brucella agar and was inoculated (10^6 bacteria/spot) on MIC assay agar plates as described previously [28]. Since a lower inoculum size, such as 10^4 bacteria/spot, causes failure of the MIC assay due to decreased viability of *H. pylori* during the preparation of many bacterial suspensions for assay, the higher inoculum size (10^6 bacteria/spot) was utilized. There were no differences between the low (10^4 bacteria/spot) and high (10^6 bacteria/spot) inoculum sizes regarding MIC of catechins tested. The incubation was performed at 37°C for 4–5 d in a microaerobic atmosphere.

Growth in liquid medium. The reference strain (ATCC 43504) and a clinical isolate (number 18) of antibiotic highly resistant *H. pylori* (MICs of CAM and MTZ: >100 μg/ml) were used in the liquid culture study. Growth of bacteria in liquid medium with or without catechins and/or antibiotics was performed using brucella broth (Difco) supplemented with 10% fetal calf serum (FCS) in a tissue culture flask with a vented filter cap (Costar, Cambridge, MA). The flasks were incubated with shaking (100 rpm) under the microaerobic condition generated by a Pack-Campylo in a rectangular jar at 37°C. The number of viable bacteria was determined on 7% horse blood-brucella agar plates after serial dilution of sample. The number of colony-forming units (CFU) was assessed after 4–5 d culture of agar plates at 37°C in a microaerobic atmosphere.

Combination effect of antibiotics and EGCg in agar medium. The combination effect of antibiotics and EGCg was determined by checkerboard assay and evaluated using fractional inhibitory concentration (FIC) index as described previously [4]. In brief, blood-brucella agar plates containing twofold serial dilutions of EGCg and/or antibiotics were inoculated with bacteria (10^6 bacteria/spot) and incubated for 4 d at 37°C in a microaerobic atmosphere. The lowest concentration at which no visible growth occurred was recorded to be the MIC value of the individual and combined EGCg and antibiotics. The FIC index was calculated from the both FIC values of EGCg and antibiotics [4]. The FIC indexes ≤ 0.5, $0.5 < \text{FIC} \leq 1$, $1 < \text{FIC} \leq 2$, and >2 were defined as synergistic, additive, indifferent, and antagonism, respectively [14].

Statistics. Statistical analysis was performed by Student *t*-test. A p value <0.05 was considered to be significant.

Results

Anti-*H. pylori* activity of tea catechins. The in vitro antibacterial activity of tea catechins against clinical isolates of *H. pylori* was examined using antibiotic-sensitive, which had ranges of MIC of AMX, CLR and MTZ of <0.03–0.5 μg/ml, <0.03–2 μg/ml, and 1–16 μg/ml, respectively, and resistant isolates, which were resistant to either or both MTZ and CLR and the ranges of MIC of MTZ and CLR of 3.12–>100 μg/ml and 0.013–100 μg/ml, respectively. Seven antibiotic-resistant isolates were resistant to both MTZ (MIC: 25–>100 μg/ml) and CLR (50–100 μg/ml). As shown in Table 1, both ECg and EGCg possessed an antibacterial activity against all clinical isolates of *H. pylori* regardless of the sensitivity of the isolates to antibiotics. The MIC of both ECg and EGCg at 90% (MIC_{90}) of all isolates was 100 μg/ml. Furthermore, there was no significant difference in terms of MIC range of both ECg and EGCg between

Table 1. MICs of catechins and antibiotics against clinical isolates of *H. pylori*

	MIC (μg/ml)					
	50% of tested isolates		90% of tested isolates		Range	
Compound	Sensitive[a]	Resistant[b]	Sensitive	Resistant	Sensitive	Resistant
C	>200	>200	>200	>200	>200	100–>200
EC	>200	>200	>200	>200	>200	>200
GC	>200	>200	>200	>200	>200	100–>200
EGC	>200	>200	>200	>200	100–>200	100–>200
ECg	100	100	100	100	50–100	25–100
EGCg	50	100	100	100	12.5–100	25–100
MTZ	8	50	16	>100	1–16	3.12–>100
CLR	0.12	50	2	100	<0.03–2	0.013–100
AMX	0.06	0.06	0.25	6.25	<0.03–0.5	0.013–6.25

[a] Thirty-seven antibiotic-sensitive clinical isolates of *H. pylori*, which were susceptible to either MTZ, CLR, or AMX, were tested.
[b] Nineteen antibiotic highly resistant (both or either MIC of MTZ, >25 μg/ml and/or MIC of CLR, >12.5 μg/ml) clinical isolates were tested.
C, catechin; EC, epicatechin; GC, gallocatechin; EGC, epigallocatechin; ECg, epicatechin gallate; EGCg, epigallocatechin gallate; MTZ, metronidazole; CLR, clarithromycin; AMX, amoxicillin.

antibiotic-sensitive versus resistant clinical isolates. In contrast, other forms of catechin did not show any antibacterial activity against *H. pylori* at the concentrations tested. The same result was also evident in the experiment with the reference strain (ATCC 43504) of *H. pylori* (data not shown).

The kinetic study of antimicrobial activity in liquid cultures. In order to analyze the kinetics of the antibacterial activity of EGCg, which has also been shown to be the most active form of tea catechins in terms of antimicrobial activity to other microorganisms [7], the effect of EGCg on growth of *H. pylori* in liquid medium was examined. The reference strain (ATCC 43504; MIC of AMX, CLR and MTZ being 0.06 μg/ml, 0.12 μg/ml, and 25 μg/ml, respectively) and an antibiotic-resistant clinical isolate (number 18; MIC of AMX, CLR, and MTZ being 0.06 μg/ml, > 100 μg/ml, and > 100 μg/ml, respectively) were cultured in FCS-brucella broth medium in the presence or absence of 25–100 μg EGCg/ml for 48 h with shaking at 37°C in a microaerobic atmosphere. The results of bacterial growth are shown in Fig. 1. As little as 50 μg/ml of EGCg, which was a half MIC, showed a strong inhibition of the growth of the reference strain (Fig. 1A). The inhibition was much stronger at 48 h after incubation. Similar results were observed when the antibiotic-resistant isolate number 18 was utilized as a target bacterium (Fig. 1B).

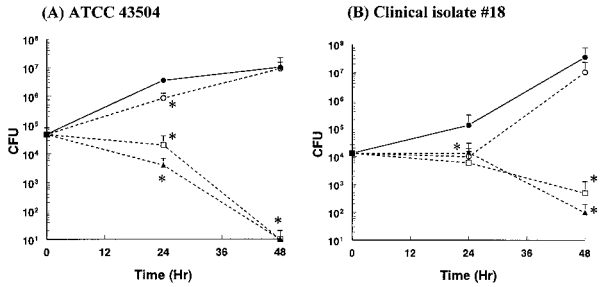

Fig. 1. Effect of EGCg on the growth of reference and highly antibiotic-resistant *H. pylori*. The reference (ATCC 43504) and antibiotic-resistant (clinical isolate number 18) *H. pylori* were cultured in brucella broth supplemented with 10% FCS in the presence or absence of the indicated concentrations of EGCg for 48 h at 37°C with shaking in a microaerobic atmosphere. The viable number of bacteria (CFU) was measured on brucella agar containing 7% horse blood. The data (means plus standard deviations from three cultures) presented are representative of three experiments: ●, control; ○, EGCg 25 μg/ml; □, EGCg 50 μg/ml; ▲, EGCg 100 μg/ml; *, $p < 0.05$ compared to the control group at the same time point.

Combination effect of EGCg with antibiotics. Determination of the possible combination of EGCg with antibiotics, which is commonly used in patients infected with *H. pylori* for eradication therapy, is appropriate, since EGCg is consumed daily as a part of tea as a beverage. Furthermore, EGCg showed antibacterial activity against antibiotic-resistant *H. pylori*, similar to antibiotic-sensitive bacteria. In order to determine the possible combination effect between EGCg and antibiotics, the reference strain of *H. pylori* was cultured with or without sub-MIC doses of EGCg (from one-fourth to one-eighth MIC) and AMX (one-fourth MIC). As shown in Fig. 2, the growth of *H. pylori* was markedly restricted in the combination group, even with as little as 12.5 μg/ml EGCg (one-eighth MIC), compared with EGCg or AMX only groups, which did not show any significant inhibition. The combination effect on bacterial growth appeared to be greater at the higher EGCg concentration,

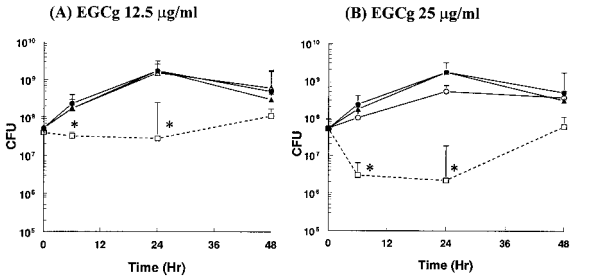

Fig. 2. Effect of EGCg in combination with AMX on the growth of *H. pylori*. *H. pylori* (ATCC 43504) cultured in brucella broth supplemented with 10% FCS in the presence or absence of indicated concentrations of EGCg and/or AMX for 48 h at 37°C in a microaerobic atmosphere. The viable number of bacteria (CFU) was measured on brucella agar containing 7% horse blood. The data (means plus standard deviations for three cultures) presented are representative of three experiments. ●, control; △, EGCg 12.5 μg/ml (A) or ○, 25 μg/ml (B); ▲, AMX 0.015 μg/ml; □, EGCg 12.5 μg/ml (A) or 25 μg/ml (B) + AMX 0.015 μg/ml; *, $p < 0.05$ compared to the control group at the same time point.

such as 25 μg/ml, but using EGCg alone did not induce a significant inhibition. However, the inhibition effect by the combination was decreased when the culture time was extended to 48 h.

In order to estimate the general combination effect between EGCg and other antibiotics, such as MTZ and CLR, on the antibacterial activity against antibiotic-sensitive as well as antibiotic-resistant clinical isolates, the FIC was determined by a checkerboard study. Twenty-three antibiotic-sensitive, 12 MTZ-resistant, and 14 CLR-resistant clinical isolates were utilized for this purpose. As evident in Table 2, the results obtained showed that more than 80% of all clinical isolates, which were significantly different ($p < 0.05$) from the number of isolates in indifferent and antagonism category, were susceptible to the effect of the combination EGCg and antibiotics, with additive as well as synergistic effects. For example, in the case of clinical isolate number 18, the MIC of MTZ and CLR decreased remarkably from > 100 μg/ml to 6.25 μg/ml (FIC = 0.56) and from 100 μg/ml to 12.5 μg/ml (FIC = 0.75), respectively, in the presence of 50 μg/ml EGCg, which did not show any inhibition of bacterial growth alone in the agar culture system. However, the synergistic effect (FIC < 0.5) between EGCg and antibiotics tested was observed with only two clinical isolates. There was no significant difference in susceptibility to the combination effect between antibiotic-sensitive (S) versus resistant isolates (R) (Table 2).

Discussion

Green tea contains many polyphenolic compounds, which account for up to 15% of the dry weight of green tea leaves [9]. Most of the polyphenols in green tea are flavanols, commonly known as catechins [1]. The EGCg and ECg forms account for about 36% and 24%, respectively, in all tea catechins [27]. Therefore, EGCg is the major component of green tea catechins. For example, a cup of green tea (150 ml) contains approximately 260 mg of EGCg. That is, the concentration of EGCg in an average individual's stomach after drinking tea may reach about ~1.7 mg/ml. Furthermore, it is known that EGCg is stable and persists in the stomach (more than 40% of EGCg remains in the stomach 2 h after ingestion) [8]. From such pharmacokinetic aspects of tea catechins, particularly EGCg, and its antibacterial activity, which has been demonstrated against a wide variety of microorganisms, some beneficial effects of tea on gastric diseases associated with *H. pylori* seem likely when constant drinking of tea each day is achieved. In fact, it has been reported epidemiologically that drinking green tea (10 or more cups per day) decreased the risk of gastric cancer [12, 15], which is now thought to be linked to *H. pylori* infection [18].

As shown in the present study, the anti-*H. pylori* activity of tea catechins varied in terms of form. EGCg and ECg were the most active forms of tea catechins regarding in vitro anti-*H. pylori* activity. In this regard, a study of structure-activity relationship has suggested that pylogallol and gallate substituent groups of catechin compounds are important. In fact, EGCg has both pylogallol and gallate substituent groups within its structure and shows the highest antimicrobial activity against a variety of microorganisms among tea catechins [10, 25]. Therefore, the results obtained in this study agreed with such previous reports and it seems conceivable that the mechanism of antibacterial activity of catechins against *H. pylori* versus other susceptible bacteria may be similar. The MIC_{90} of both EGCg and ECg against all clinical isolates of *H. pylori* was 100 μg/ml, which did not appear to be strong based on the concentration in comparison with that of antibiotics, such as MTZ, CLR, and AMX. Previous report regarding antimicrobial activity of EGCg and ECg against *H. pylori* clinical isolates showed a 32 μg/ml of MIC_{90} [17], which is stronger than the MICs observed here. Such different MIC values may be due to different MIC assay method employed (broth dilution method versus agar dilution method). Nevertheless, this level of EGCg MIC can be readily reached in the stomach after drinking a cup of green tea. Furthermore, the study also showed that these catechins were effective against highly antibiotic (MTZ and/or CLR) resistant clinical isolates of *H. pylori*.

The kinetic study of the antibacterial activities revealed the slow-acting nature of EGCg. That is, the apparent antibacterial activity was not observed until

Table 2. Combination effect of EGCg and antibiotics against *H. pylori*

Combination	Isolates[a]	Number of clinical isolates			
		Synergistic FIC ≤ 0.5	Additive 0.5 <FIC ≤ 1	Indifferent 1 < FIC ≤ 2	Antagonism FIC > 2
EGCg + MTZ	S (5)	1	4	0	0
	R-MTZ (12)	0	9	3	0
EGCg + CLR	S (11)	0	9	2	0
	R-CLR (14)	1	9	4	0
EGCg + AMX	S (7)	0	7	0	0
Subtotal	S (23)	21 (91.3%)		2 (8.7%)	
	R (26)	19 (73.0%)		7 (26.9%)	
Total	(49)	40 (81.6%)		9 (18.3%)	

[a] Number in parenthesis shows the number of isolates tested.
S, antibiotic-sensitive isolates; R-MTZ, metronidazole-resistant (MIC ≥ 25 μg/ml) isolates; R-CLR, clarithromycin-resistant (MIC ≥ 50 μg/ml) isolates.

24 h after cultivation. A decline in CFU after 24 h cultivation indicates a delayed killing activity of EGCg on *H. pylori*. Similar results were also observed in experiments with an antibiotic-resistant clinical isolate. These results indicate that the anti-*H. pylori* activity of EGCg may require a certain time period for contact with the target bacteria, but this green tea component inhibits growth of all *H. pylori* organisms regardless of their susceptibility to antibiotics. However, the mechanism of the antibacterial activity of EGCg on *H. pylori* is not yet known, but nonspecific membrane perturbation may account for one of the mechanisms, as is the case with some other bacteria [7].

Since the anti-*H. pylori* activity of EGCg was not remarkable and required a prolonged contact time period for expression of antibacterial activity against *H. pylori*, eradication of *H. pylori* by EGCg alone in experimental animal models has not succeeded, even though remarkable improvement of pathological findings was noted [17]. In the present study, the combination effect of EGCg and antibiotics on the growth of *H. pylori* in liquid cultures was shown in terms of antibacterial activity. That is, as little as 12.5 μg/ml EGCg enhanced the antibacterial activity of the antibiotic AMX, which did not show any antibacterial activity against bacteria at the concentration used. However, such combination effect was decreased at the late phase of incubation, such as 48 h after culture. The reason for the decrease is not clear. Stability of the antibiotic in the culture at the late phase may be possible. The general combination effect of EGCg and other antibiotics, such as MTZ and CLR, against clinical isolates including antibiotic-resistant isolates was also evident in this study. However, such combination effect did not always succeed against all clinical isolates tested. Even though more than 80% of the clinical isolates tested were susceptible to the combination effect, some isolates were resistant to the combination effect. No relation was observed between the degree of resistance to the antibiotic and the combination effect (data not shown). The reason for such variation of susceptibility between clinical isolates of *H. pylori* to the combination effect is not clear. The mechanism for the combination effect is also not known, even though this effect was not synergistic but additive with a majority of clinical isolates. Since the antibacterial mechanisms of two antibiotics MTZ and CLR are quite different [19], a common mechanism for the combination effect between EGCg and these antibiotics may not exist. Nevertheless, the present results warrant further study regarding the mechanism(s) of the combination effect between EGCg and antibiotics.

In summary, the tea catechin EGCg showed an obvious in vitro anti-*H. pylori* activity against both antibiotic-sensitive and resistant clinical isolates. The inhibition activity was slow but nevertheless strong on the *H. pylori* growth. In combination with antibiotics, EGCg enhanced the antibacterial activity of antibiotics against most clinical isolates tested, regardless of their antibiotic resistance. These results indicate that tea catechin EGCg may be a possible potential alternative agent to treat gastric diseases associated with *H. pylori* infection, particularly in combination with antibiotics.

ACKNOWLEDGMENTS

The authors thank Dr. Herman Friedman, University of South Florida College of Medicine, Tampa, FL for his critical review of this manuscript. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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