Antimutagenic Effect of Epigallocatechin Gallate and its Effect on the Immune Response in Mice

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Abstract

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Green tea is the second-most consumed beverage in the world (water is the first one) and has been used medicinally for centuries in India and China. The active substances in the green tea are polyphenols (catechins) and flavonols which possess a potent antioxidant activity. Epigallocatechin gallate (EGCG) is one of the four major green tea catechins. Using the Ames test, micronucleus test, comet assay, chemiluminescence test, and blastic transformation test, we examined the antimutagenic effects of chemoprotective substance epigallocatechin gallate (EGCG) in the pure form on the mutagenicity induced by three reference mutagens: aflatoxin B_1 (AFB_1), 2-amino-3-methylimidazo [4,5-f] quinoline (IQ), and *N*-nitroso-*N*-methylurea (MNU), and the effect of EGCG on the immunosuppression caused by these mutagens. Using the Ames test the dose dependent antimutagenic activity of EGCG was proved against indirect mutagens AFB_1 and IQ, but not against the direct mutagen MNU. In the micronucleus test, EGCG had antimutagenic effect upon all three mutagens. EGCG decreased the level of DNA breaks induced by AFB_1 in bone marrow cells and colon epithelium, and the level of DNA breaks induced by MNU in colon cells to the level found in control. The reparatory effect of EGCG on immunosupression induced by all three carcinogenic compounds was proved using chemiluminescence and blastic trasformation tests.

Keywords: epigallocatechin gallate; antimutagenic effects; effect on the immune response; Ames test; micronucleus test; comet assay; chemiluminescence and blastic transformation tests

The exposure of the population to carcinogenic substances represents an important factor in the incidence of tumour diseases. In economically developed countries, tumour diseases are the second most frequent cause of death (Stratil & Kubáň 2004). There are significant differences in the cancer incidence and mortality among ethnic groups who have different lifestyles and have

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been exposed to different environmental factors (American Cancer Society 2003).

On the other hand, there are various kinds of vegetables, fruit and other plants with anti-carcinogenic, anti-mutagenic, immunostimulatory or immunomodulatory effects. There is increasing evidence that dietary phytochemicals may play important roles as chemopreventive or chemotherapeutic agents in the prevention of many diseases, including tumours. If chemopreventive agents are components of our daily food or drink, it will be fortunate to human beings.

Tea (*Camellia sinensis*), a tropical shrub originated in Southern China, is consumed by over two thirds of the world's population. The Chinese used tea as a medicinal drink as early as 3000 BC, and by the end of the sixth century as a beverage. The polyphenols are the most important chemicals present in tea, with considerable pharmacological significance. Among the most extensively investigated and well-defined dietary chemopreventives, polyphenol (–)-epigallocatechin-3-gallate (EGCG) is a principal antioxidant contained in green tea.

Multiple lines of evidence from epidemiologic studies indicate that frequent consumption of green tea is inversely associated with the risk of several types of human cancer (Fujiki *et al*. 2002; Park & Surh 2004). It has been reported by many researchers that the hot water extract of green tea and green tea polyphenols have anti-mutagenic activity (Weisburger *et al.* 1996) or suppress the tumour promotion. A pilot study with 8552 subjects from Saitama Prefecture in Japan has revealed that green tea has a potentially preventive effect against cancers of all organs including stomach, lung, colorectum, and liver (Nakachi *et al*. 2000).

Numerous EGCG studies demonstrated chemopreventive and chemotherapeutic actions in cellular and animal models of cancer. EGCG selectively induces apoptosis in human carcinoma cell lines (Borska *et al.* 2003; Lambert & Yang 2003; Gupta *et al.* 2003, 2004). It inhibits MAP kinase mediated signalling pathways (Sah *et al*. 2004). EGCG blocks the activation of EGF receptors and HER-2/neu oncogen which is over-expressed or constitutively active in many human malignancies (Masuda *et al.* 2003). EGCG interferes with angiogenesis by suppressing VEGF activity, VE-cadherin phosphorylation and matrix metalloproteinase activity (Lee *et al.* 2004), inhibits metastatic spreading of the adenocarcinoma of mouse prostate Sartor *et a*l. (2004), inhibits benzo[*a*]pyrene and cisplatin-induced lung tumorigenesis in mice (Banerjee *et al.* 2005). EGCG inhibits DNA methyltransferase and telomerase, two enzymes involved in the cancer gene expression and cellular immortality (Yokoyama *et al.* 2004; Didiano *et al.* 2004; Mittal *et al*. 2004), and play a role in the protection against free-radical DNA damage (Saffari & Sadrzadeh 2004; Sugisawa *et al.* 2004).

Recent studies have shown that the tea polyphenol catechins have various physiologic modulative activities, such as antioxidative effect (Lee *et al.* 2003) and antibacterial effect (Amarowicz *et al.* 2000).

The results of Alvarez *et al.* (2002) show that epigallocatechin gallate is capable to modulate reactive oxygen species production during the respiratory burst of rat peritoneal macrophages by acting as a superoxide anion scavenger. EGCG may therefore be useful in the prevention and treatment of diseases which are due to an increased free radical production. Tea catechins treatment significantly increased cell viability and decreased the lipid peroxidation levels (Chen *et al.* 2002), inhibited the release of arachidonic acid from cells (Lu *et al.* 2003) and suppressed the proliferation of human vascular smooth muscle cells exposed to high levels of native LDL (Locher *et al.* 2002). EGCG caused a significant induction of cell cycle arrest and apoptosis of melanoma cells (Nihal *et al.* 2005)

In the present paper, the effect was studied of epigallocatechin gallate in the pure form on the mutagenicity or immunosuppression caused by three known mutagens: aflatoxin B_1 (AFB1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and *N*-nitroso-*N*-methylurea (MNU) using the Ames bacterial mutagenicity test and *in vivo* micronucleus, chemiluminescence, and blastic transformation tests, resp. comet assay.

In our study, we chose different representants of mutagens which may occur in the human diet – two promutagens – mycotoxin AFB_1, food mutagen IQ, and MNU, the direct mutagen of endogenous origin.

MATERIAL AND METHODS

Chemicals. The following chemicals were used in all tests: AFB_1 (Alexis Corporation, USA), IQ

(ICN Biomedicals, Inc., Germany), MNU (Sigma-Aldrich Co, Lousiana, USA), epigallocatechin gallate (Sigma-Aldrich Co, Lousiana, USA). Chemicals were diluted with dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co, Lousiana, USA).

Ames test. The Ames test with *Salmonella typhimurium* TA98 and TA100 (Ames *et al.* 1975; Černá *et al*. 1989) was used for the evaluation of the antimutagenic effect of epigallocatechin gallate *in vitro*.

The mutagenic substances were used at the following concentrations: AFB_1 at concentrations 10 μg, 1 μg, and 0.1 μg per plate with both strains TA98 and TA100, IQ at concentrations 0.1 μg, 0.01 μg, and 0.001 μg per plate with the strain TA98, at concentrations 10 μg, 1 μg, and 0.1 μg with the strain TA100, MNU at concentrations 1000 μg, 100 μg, and 10 μg only with the strain TA100 because these concentrations had no effect on the strain TA98. The individual concentrations of each mutagen were combined with four different concentrations of the antimutagen (300 μg, 30 μg, 3 μg, and 0.3 μg of epigallocatechin gallate per plate). All chemicals were diluted with DMSO. For the metabolic activation of AFB_1 and IQ, the S9 liver homogenate fraction from laboratory rats influenced by the mixture of polychlorinated biphenyls Delor was used (Maron & Ames 1983). The combinations of mutagen and antimutagens were tested in two separate experiments with three plates in each experiment.

Percentage of the inhibition of mutagenicity was calculated (Šmerák *et al.* 2006).

For the evaluation of differences between the means of revertant numbers in the samples with a mixture of mutagen and antimutagen, and in samples with mutagen only, unpaired Student's *t*-test was used.

Experimental animals. *In vivo* experiments (bone marrow micronucleus test) were carried out on ten-week-old male Balb C mice, weighing 22–26 g (purchased from BIOTEST, Konárovice, CZ). The animals were housed under the controlled light regime of 12/12 h, temperature of 20 ± 2°C, relative humidity of 60 ± 10%, and complete air recirculation 10–14-times per hour. The animals were supplied with water *ad libitum* and were fed with a commercial granulated mixture for laboratory rodents. These animals were divided into groups of 10 mice each.

Micronucleus test. The mouse bone marrow micronucleus test was carried out according to Schmid (1975). An increased frequency of micronuclei in polychromatophilic erythrocytes in comparison with the control groups indicates that the substance tested induces chromosomal damage in nucleated erythrocytes in the bone marrow. A total of 1000 polychromatophilic erythrocytes were scored per animal by the same observer for evaluating the frequencies of micronucleated polychromatophilic erythrocytes. Each experiment was run three times. Control mice were treated orally by a 7% solution of DMSO. For the statistical analysis of the differences between the micronucleus values in the animals treated with a mixture of mutagens and EGCG and the animals treated with mutagen alone, unpaired Student's *t*-test was used.

Single strand breaks – Comet assay. The alkaline version of the comet assay according to Singh *et al*. (1988) and modified by Collins *et al*. (1997) was used. Cells were embedded in agarose on a microscope slide, lysed with a nonionic detergent and left to unwind the DNA in the alkaline electrophoresis solution (0.3M NaOH, 10mM EDTA) for 40 min. Electrophoresis (25 V, 300 mA, 4°) results in the attraction of DNA to the anode. However, if the DNA is intact, the supercoiling prevents any significant movement. The relative amount of DNA in the tail of the comet formed reflects the number of breaks in the DNA. The per cent of DNA in tail was converted to the number of SSB/10^9 Da using the calibration of the method by X-ray irradiation (Collins *et al*. 1996). For the statistical significance evaluation of the difference between the animals treated with a mixture of mutagens and EGCG and the animals treated with mutagen alone, Mann-Whitney *U* test was used.

Chemiluminescence test. The chemiluminescence test was performed using the modification by Šestáková *et al*. (1997). In the chemiluminescence test, the degree to which phagocytes are capable to liquidate the ingested material is determined. Well known are the mechanisms in which hydrogen peroxide participates in killing (Klebanoff 1980). H_2O_2 is synthetised in phagocytes upon receiving a signal by their membranes. The finding of a higher or lower activity of the complex H_2O_2-MPO-Cl^- (I^-) is connected with the readiness of the first defense line against bacteria, tumour cells, as well as carcinogen-altered cells. The results are presented in maximum values (mV) of the chemiluminescence response of polymorphonuclear leucocytes in the dependence on time.

The blastic transformation method. For the study of the readiness of cells in acquired immunity we used a functional test assessing T-lymphocytes, the blastic transformation method (Field 1996). This test of lymphocyte activation determines the functional capacity of T-lymphocytes to respond to a mitogen by proliferation, and as such it is a more direct examination of the immune competence than just determining the numbers of various lymphocyte populations (Stites & Terr 1994). For the statistical analysis, paired Student's *t*-test was used for both immunologic tests.

Substances used for in vivo tests. The following concentrations of the substances tested were used in *in vivo* tests – the micronucleus test and comet assay: AFB_1 5 mg per kg of body weight (b.w.), IQ 20 mg/kg b.w., MNU 50 mg/kg b.w., epigallocatechin gallate 10 mg/kg b.w. In the chemiluminescence test and the blastic transformation method were used: AFB_1 1 mg/kg b.w., IQ 20 mg/kg b.w., MNU 20 mg/kg b.w. Epigallocatechin gallate was administered to mice at the dose of 10 mg/kg b.w. by gavage for three sequential days. Carcinogens were administered in one dose on the third day. The controls received 7% DMSO. All the substances (diluted with DMSO) were administered in volumes of 100 µl/10 g b.w.

RESULTS

The Ames test

The results of the Ames test are presented in Tables 1–3 as a number of revertants and as the percentage of inhibition of the mutagenic activity of the sample of the tested mutagen together with antimutagen in relation to the mutagenic activity of the tested mutagen alone. Two highest

Table 1. Effect of EGCG on mutagenicity of AFB_1 – Ames test – TA98, TA100

AFB_1 + EGCG dose (µg/plate)	*S. typhimurium* TA98 + S9			*S. typhimurium* TA100 + S9		
	No. of revertants	± SD	% inhibition	No. of revertants	± SD	% inhibition
10 + 0	1018	84		1231	231	
10 + 0.3	860*	75	–16	1139	230	–8
10 + 3	745**	91	–27	1098	200	–11
0 + 30	230**	19	–77	446**	74	–64
10 + 300	96**	14	–91	206**	30	–83
1 + 0	366	38		612	137	
1 + 0.3	286*	48	–22	577	134	–6
1 + 3	209**	12	–43	488	52	–20
1 + 30	77**	11	–79	207**	16	–66
1 + 300	47**	6	–87	104**	19	–83
0.1 + 0	86	15		205	50	
0.1 + 0.3	84	9	–2	211	13	+3
0.1 + 3	82	14	–5	192	28	–6
0.1 + 30	39**	5	–55	123*	14	–40
0.1 + 300	34**	6	–61	100**	5	–51
Control (DMSO)	25	5		95	11	
0 + 0.3	27	2		87	4	
0 + 3	27	2		95	4	
0 + 30	18	2		92	9	
0 + 300	25	8		90	8	

SD – standard deviation

Statistically significant difference between the sample with EGCG and mutagen and the sample with mutagen alone *$P \leq 0.05$ and ** $P \leq 0.01$

Table 2. Effect of EGCG on mutagenicity of IQ – Ames test – TA98, TA100

IQ_ + EGCG dose (µg/plate)	*S. typhimurium* TA98 + S9			IQ_ + EGCG dose (µg/plate)	*S. typhimurium* TA100 + S9		
	No. of revertants	± SD	% inhibition		No. of revertants	± SD	% inhibition
0.1 + 0	732	194		10 + 0	692	104	
0.1 + 0.3	760	218		10 + 0.3	687	126	–1
0.1 + 3	755	156	+3	10 + 3	653	103	–6
0.1 + 30	168**	32	–77	10 + 30	185**	48	–73
0.1 + 300	36 **	10	–95	10 + 300	79**	14	–89
0.01 + 0	177	48		1 + 0	229	30	
0.01 + 0.3	186	99	+5	1 + 0.3	229	25	0
0.01 + 3	174	77	–2	1 + 3	214	28	–7
0.01 + 30	41**	8	–77	1 + 30	111**	8	–52
0.01 + 300	35**	5	–80	1 + 300	91**	16	–60
0.001 + 0	59	12		0.1 + 0	115	9	
0.001 + 0.3	62	23	+5	0.1 + 0.3	116	6	+1
0.001 + 3	59	10	0	0.1 + 3	110	11	–4
0.001 + 30	23**	3	–61	0.1 + 30	90**	12	–22
0.001 + 300	32**	5	–46	0.1 + 300	87**	8	–24
Control (DMSO)	20	5		Control (DMSO)	77	5	
0 + 0.3	26	4		0 + 0.3	84	4	
0 + 3	20	5		0 + 3	91	5	
0 + 30	18	5		0 + 30	96	9	
0 + 300	20	5		0 + 300	78	11	

SD – standard deviation

Statistically significant difference between the sample with EGCG and mutagen and the sample with mutagen alone $^{**}P \leq 0.01$

concentrations of EGCG (300 and 30 µg/plate) revealed a significant inhibition of the mutagenic effects with all concentrations of both indirect mutagens AFB_1 and IQ in the strains TA98 and TA100 (Tables 1 and 2). This effect was dose dependent and was the highest at the concentration of 300 µg/plate. Lower concentrations of EGCG (3 and 0.3 µg/plate) were effective only against two concentrations of AFB_1 (10 and 1 µg/plate) in TA98 strain (Table 1).

EGCG was without any effect at all in the combinations with the direct mutagen MNU (Table 3). A weak decrease of the mutagenic activity was detected only with the highest concentration of EGCG combined with the lowest concentration of MNU (10 µg/plate), but this effect was not significant.

The micronucleus test

The number of micronuclei in the animals influenced by epigallocatechin gallate alone did not differ from that of the control group. On oral administration of the combinations of three doses of epigallocatechin gallate (10 mg/kg) and a single dose of aflatoxin B1 (5 mg/kg), the number of micronuclei in polychromatophilic erythrocytes was lowered to a statistically significant degree in comparison with the laboratory mice treated with AFB1 alone. A similar effect was observed on the administration of IQ mutagen: epigallocatechin gallate in combination with IQ mutagen (3 × 10 mg/kg + 20 mg/kg) reduced its mutagenic effect to a statistically significant degree. Similarly, the treatment of mice with a combination of

Table 3. Effect of EGCG on mutagenicity MNU – Ames test – TA100

MNU + EGCG dose (µg/plate)	*S. typhimurium* TA100			MNU + EGCG dose (µg/plate)	*S. typhimurium* TA100		
	No. of revertants	± SD	% inhibition		No. of revertants	± SD	% inhibition
1000 + 0	1876	176		10 + 0	843	209	
1000 + 0.3	1893	217	+1	10 + 0.3	843	143	0
1000 + 3	1895	165	+1	10 + 3	879	168	+4
1000 + 30	1843	198	–2	10 + 30	840	180	0
1000 + 300	1875	233	0	10 + 300	648	104	–23
100 + 0	1923	168		Control (DMSO)	64	16	
100 + 0.3	1961	198	+2	0 + 0.3	77	17	
100 + 3	1920	126	0	0 + 3	70	25	
100 + 30	1960	139	+2	0 + 30	68	24	
100 + 300	1953	154	+2	0 + 300	63	28	

SD – standard deviation

epigallocatechin gallate and MNU (3 × 10 mg/kg + 50 mg/kg) led to a significant reduction of the number of micronuclei in comparison with the number of micronuclei induced by MNU alone. The results are presented in Table 4.

Table 4. Numbers of micronuclei in polychromatophilic erythrocytes in the bone marrow of mice treated with combinations of epigallocatechin gallate (EGCG) and the mutagens

Substance studied	No of micronuclei	± SD
Control 7% DMSO	1.5**	1.0
EGCG 10 mg/kg	1.7**	2.3
AFB_1 5 mg/kg	11.3*	2.4
EGCG + AFB_1 10 mg/kg + 5 mg/kg	5.0**	0.75
IQ 20 mg/kg	11.0*	2.45
EGCG + IQ 10 mg/kg + 20 mg/kg	4.8**	1.17
MNU 50 mg/kg	17.0*	1.9
EGCG + MNU 10 mg/kg + 50 mg/kg	12.4**	3.2

SD – standard deviation

*significantly higher number of micronuclei as against the negative control (DMSO)

**significantly lower number of micronuclei as against mutagen alone

Comet assay

We measured the induction of DNA breaks in liver, bone marrow and colon epithelium cells of the mice pre-treated with EGCG for three days before the mutagen administration. The amount of SSB was measured in the cells isolated from the particular tissue, 2 h after the application of the mutagen, by means of the comet assay. No significant induction of DNA breaks in any of the organs followed was observed after the IQ application in the dose used in this study. A significant induction of DNA breaks was found in bone marrow and colon epithelium cells after the administration of AFB_1. Surprisingly, AFB_1 did not induce any DNA breaks in liver cells (Table 5). AFB_1 induced DNA breaks both in bone marrow and colon epithelium cells. The level of DNA breaks increased from 0.9 to 1.6 SSB/10^9 Da in bone marrow cells, and from 2.2 to 3.2 SSB/10^9 Da found in colon cells. EGCG administered for 3 days before AFB_1 decreased the amount of DNA breaks induced by AFB_1 to the level found in control animals. This decrease was statistically significant.

Similar results were observed with MNU (Table 6). This mutagen increased the amount of DNA breaks significantly from 0.5 to 0.95 SSB/10^9daltons in bone marrow cells, from 0.5 to 1.2 SSB/10^9 Da in liver cells, and from 1.8 to 3.2 SSB/10^9 Da in colon epithelium cells. While EGCG caused only

Table 5. Numbers of DNA breaks in the bone marrow (BM), liver and colon of mice treated with a combination of epigallocatechin gallate (EGCG) and AFB_1

	Control	SE	EGCG	SE	AFB_1	SE	EGCG + AFB_1	SE
BM	0.88	0.06	0.70	0.04	1.58	0.35	0.98*	0.10
Liver	0.52	0.05	0.60	0.12	0.72	0.13	0.75	0.10
Colon	2.20	0.13	2.41	0.36	3.21	0.21	2.02*	0.30

*significantly lower number of DNA breaks as against mutagen alone

Table 6. Numbers of DNA breaks in the bone marrow (BM), liver and colon of mice treated with a combination of epigallocatechin gallate (EGCG) and MNU

	Control	SE	EGCG	SE	MNU	SE	EGCG + MNU	SE
BM	0.48	0.05	0.45	0.09	0.95	0.07	0.80	0.03
Liver	0.41	0.0	0.40	0.05	1.22	0.10	1.05	0.10
Colon	1.84	0.17	1.92	0.17	3.24	0.16	2.01*	0.40

*significantly lower number of DNA breaks as against mutagen alone

a small, insignificant decrease of DNA breaks in bone marrow and liver cells, it decreased the amount of DNA breaks in colon cells to the level found in controls.

Determination of test chemiluminescence

In the experiment with AFB_1 administered to mice together with EGCG, in the group treated with EGCG only chemiluminescence values have been found to be higher to a statistically significant degree as compared to control animals (days 1 and 7, $P < 0.01$; days 12 and 17, $P < 0.05$). On the 25^{th} day, the values of both groups of animals did not differ significantly. Likewise, the group of mice treated with AFB_1 + EGCG revealed significantly increased chemiluminescence values over almost the whole period followed, in comparison with the control group (days 1 and 17, $P < 0.05$; days 7 and 12, $P < 0.01$; day 25, N.S.). Over the whole period of the follow-up, EGCG alone or in combination with AFB_1 repaired the suppressive effect of the carcinogen in the lethal phase of phagocytosis in murine granulocytes (Table 7).

In the experiment with IQ and EGCG, in animals treated with EGCG only a statistically significant increase was found in the value of chemiluminescence over the duration of the experiment as compared to controls ($P < 0.01$; day 26, $P < 0.05$). The same results were obtained with the group of mice treated with IQ + EGCG. In the course of testing, the latter in combination with IQ repaired the suppression of phagocytosis in murine granulocytes caused by the carcinogen IQ (Table 8).

In the experiment with MNU and EGCG, significantly higher values of chemiluminescence

Table 7. Chemiluminescence test – values in mV after the administration of EGCG and AFB_1

Days after administration of mutagen	Control 7% DMSO ± S.D.	AFB_1 1 mg/kg ± S.D.	EGCG 3× 10 mg/kg ± S.D.	EGCG + AFB_1 ± S.D.
1	518 ± 10.6	310 ± 8.0	686 ± 13.6	455 ± 12.5
7	530 ± 11.2	262 ± 6.2	690 ± 10.2	798 ± 14.4
12	515 ± 10.0	244 ± 5.8	625 ± 12.7	766 ± 13.8
17	522 ± 12.8	203 ± 4.0	588 ± 9.2	624 ± 10.2
25	527 ± 13.6	194 ± 3.7	500 ± 10.0	482 ± 10.6

Table 8. Chemiluminescence test – values in mV after the administration of EGCG and IQ

Days after administration of mutagen	Control 7% DMSO ± S.D.	IQ 20 mg/kg ± S.D.	EGCG 3 × 10 mg/kg ± S.D.	EGCG + IQ ± S.D.
1	415 ± 12.2	339 ± 8.3	610 ± 12.4	585 ± 13.5
7	428 ± 10.8	298 ± 8.7	599 ± 11.0	687 ± 12.4
12	419 ± 9.1	266 ± 10.2	570 ± 12.3	653 ± 10.8
19	408 ± 8.6	189 ± 6.5	545 ± 9.4	618 ± 11.9
26	400 ± 10.9	157 ± 6.0	487 ± 10.6	504 ± 10.9

Table 9. Chemiluminescence test – values in mV after the administration of EGCG and MNU

Days after administration of mutagen	Control 7% DMSO ± S.D.	MNU 20 mg/kg ± S.D.	EGCG 3 × 10 mg/kg ± S.D.	EGCG + MNU ± S.D.
1	500 ± 12.5	280 ± 9.6	656 ± 13.5	465 ± 10.9
6	496 ± 11.8	266 ± 8.3	660 ± 10.2	568 ± 12.5
12	503 ± 10.9	245 ± 7.4	614 ± 12.5	560 ± 10.9
19	508 ± 12.0	200 ± 7.5	598 ± 11.4	558 ± 9.2
26	499 ± 11.3	165 ± 8.0	550 ± 10.6	526 ± 10.5

Table 10. Blastic transformation test – values in SI after the administration of EGCG and AFB_1

Days after administration of mutagen	Control 7% DMSO ± S.D.	AFB_1 1 mg/kg ± S.D.	EGCG 3 × 10 mg/kg ± S.D.	EGCG + AFB_1 ± S.D.
6	17.16 ± 3.45	7.63 ± 1.55	15.52 ± 2.99	16.70 ± 3.08
12	16.50 ± 3.7	4.64 ± 0.83	14.18 ± 2.97	12.90 ± 2.97
20	18.48 ± 4.24	5.34 ± 1.03	15.48 ± 3.28	10.02 ± 2.02

Table 11. Blastic transformation test – values in SI after the administration of EGCG and IQ

Days after administration of mutagen	Control 7% DMSO ± S.D.	IQ 20 mg/kg ± S.D.	EGCG 3 × 10 mg/kg ± S.D.	EGCG + IQ ± S.D.
6	17.16 ± 3.45	5.99 ± 0.54	15.52 ± 2.99	15.13 ± 2.80
12	16.50 ± 3.70	4.15 ± 0.44	14.18 ± 2.97	14.20 ± 2.71
20	18.48 ± 4.24	4.80 ± 0.89	15.48 ± 3.28	10.51 ± 2.32

Table 12. Blastic transformation test – values in SI after the administration of EGCG and MNU

Days after administration of mutagen	Control 7% DMSO ± S.D.	MNU 20 mg/kg ± S.D.	EGCG 3 × 10 mg/kg ± S.D.	EGCG + MNU ± S.D.
6	17.16 ± 3.45	6.72 ± 1.52	15.52 ± 2.99	16.57 ± 2.82
12	16.50 ± 3.70	4.78 ± 0.75	14.18 ± 2.97	13.36 ± 3.02
20	18.48 ± 4.24	5.19 ± 1.11	15.48 ± 3.28	11.13 ± 1.97

were found in animals treated with EGCG alone in comparison with controls (days 1 and 6, $P < 0.01$; days 12, 19 and 26, $P < 0.05$). The chemiluminescence values in animals treated with MNU + EGCG did not differ from controls on the first and last days of investigation. Significantly higher values were found in this group of mice on days 6, 12 and 19 ($P < 0.05$). Also in that experiments, EGCG alone and in combination with MNU repaired the suppressive effect of the carcinogen MNU in the lethal phase of phagocytosis in murine granulocytes (Table 9).

Determination of blastic transformation

In all the experiments over the whole period of study each respective carcinogen significantly suppressed the SI value ($P < 0.01$) in comparison with the combinations of EGCG and carcinogens. In the experiments with three different carcinogens, on no day did SI values in control groups of mice differ significantly from those treated with either EGCG alone or in combination with any of the three carcinogens – AFB_1 (Table 10), IQ (Table 11), MNU (Table 12).

DISCUSSION

In our experiments with the Ames test, we found significant antimutagenic effects of EGCG against indirect mutagens AFB_1 and IQ. No significant antimutagenic activity was proved against the direct mutagenicity of MNU. Similar results were obtained by many authors in *Salmonella* tests (for a review see Gupta *et al*. 2002). Contrary to our results, Hour *et al*. (1999) revealed the supression of directly acting mutagens MNNG and MNU, the mechanism of which they explain by free radical scavenging and antioxidant ability. Muto *et al*. (2001) demonstrated that green tea polyphenols, particularly ECG and EGCG, inhibited the metabolic activation of B[*a*]P, PhIP and AFB_1 by human CYP enzymes in *Salmonella typhimurium* assay. Our results support the idea that the effect on the metabolic activation of indirect mutagens and carcinogens is dominant in the protective effect detected by *in vitro* tests.

According to our *in vivo* micronucleus test, EGCG was antimutagenic against all three mutagens used. The formation of micronuclei and DNA damage in the form of comet tail length during single cell electrophoresis was significantly suppressed by EGCG in a dose dependent manner in a study by Roy *et al*. (2003). Also Ito and Ito (2001) demonstrated the suppressive effect of EGCG on AFB_1 – induced chromosome aberrations in rat bone marrow cells. We found as well that EGCG is able to decrease the number of DNA breaks induced by AFB_1 in BM cells, and DNA breaks induced by MNU or AFB_1 in colon epithelium cells.

The anticarcinogenic effect of EGCG includes many other effects reviewed by Park and Surh (2004), Bode and Dong (2004) and Sarcar and Li (2004). Apart from the antioxidant activity, mentioned it is the modulation of cell signalling cascades with the final effect on many transcription factors, the inhibition and influence of cell cycle through the cell cycle regulation proteins. EGCG has also proapoptotic activity in transformed cells and antiangiogenic, antiinvasive, and antimetastatic potential. In antiproliferative effects of EGCG, the stimulation of a negative cell cycle regulators as p53, p57 and other proapoptotic proteins plays a decisive role.

Green tea catechins, such as epigallocatechin gallate, repress the reactive oxygen species activity, inhibit apoptosis of activated neutrophils, and dramatically inhibit chemokine-induced neutrophil chemotaxis *in vitro*, EGCG and green tea extract block neutrophil-mediated atherogenesis *in vivo* (Dona *et al*. 2003). The studies by Yamamoto *et al*. (2004a) showed that EGCG enhance the *in vitro* resistance of alveolar macrophages to *Legionella pneumophilia* infection by a selective immunomodulatory effect on cytokine formation. EGCG lowers the levels of several markers of oxidative stress, and many *in vitro* experiments support its strong antioxidant activity (Henning *et al*. 2004). Tea catechins and polyphenols are effective scavengers of reactive oxygen species *in vitro*, especially of superoxide, which play a key role in tissue damage (Buttemeyer *et al*. 2003), and of hydrogen peroxide (Yamamoto *et al*. 2004b). The catechins may also act indirectly as antioxidants, through their effects on the transcription factors and enzyme activities (Chen *et al*. 2003). EGCG can act as an antioxidant by trapping peroxyl radicals, it inhibits lipid peroxidation and is capable to protect erythrocyte membrane-bound ATPases against oxidative stress (Saffari & Sadrzadeh 2004). The bioactive ingredients in green tea extract cause an increase in the activity of glutathione peroxidase and glutathione reductase and in the content of reduced glutathione, as well as a marked

decrease in the contents of lipid hydroperoxides, 4-hydroxynonenal (4-HNE), and malondialdehyde (MDA) in the liver. Green tea protects against lipid peroxidation in the liver, blood serum, and central nervous tissue (Skrzydlewska *et al*. 2002). Locher *et al*. (2002) described that the green tea constituents inhibit the proliferation of human vascular smooth muscle cells exposed to high levels of native LDL. Green tea constituents and antioxidants may exert vascular protection by inhibiting human vascular smooth muscle cell growth associated with hypercholesterolemia. The system relevant for the prevention of carcinogenesis *in vivo* includes the modulation of drug metabolism (inhibition of Cyp 1A activity, induction of NAD(P)H:quinone reductase activity, free radical scavenging, anti-inflammatory mechanism, inhibition of cyclooxygenase-1). The effective cancer chemopreventive agents are catechins, such as epigallocatechin gallate (Gerhauser *et al*. 2003). EGCG also showed a strong inhibition of tyrosine kinase and mitogen-activated protein kinase (MAPK) activities, without affecting the kinase in normal cells. EGCG decreases the levels of the oncogen proteins in transformed cells and inhibits the proliferation of leukemic cells (Wang & Bachrach 2002).

In the present paper, we followed mice treated with epigallocatechin gallate and AFB_1, IQ, or MNU, using the chemiluminescence test (which reflects the degree of phagocytosis by murine granulocytes). After the administration of three different carcinogens, we found, in agreement with published data, a marked inhibition of phagocytosis, namely of the complex: hydrogen peroxide – myeloperoxidase – halide factor, by AFB_1, IQ, and MNU. Epigallocatechin gallate in all experiments increased the chemiluminescence values most significantly in comparison with controls. These results are in agreement with the finding of the increased luminol-enhanced chemiluminescence of white blood cells in peripheral blood stimulated by zymosan after the administration of the tea polyphenols to mice with Lewis lung carcinoma (Zhu *et al*. 1999). EGCG increased the reduction of nitro blue tetrazolium (NBT) (Alvarez *et al*. 2002). In our study EGCG also stimulated chemiluminescence, the most markedly and significantly in the combination with carcinogens.

In the blastic transformation test which is an indicator of the functional state of T-lymphocytes, a statistically significant inhibition of the blast formation in the population of carcinogen-stimulated murine spleen cells after the application of any of the three carcinogens has been registered. Our finding is in agreement with the data on the suppression of blastic transformation by AFB_1 (Reddy & Sharma 1989) and by benzo(*a*)pyrene which belongs to polyaromatic hydrocarbons (Tomar *et al.* 1991).

Our results on spleen lymphocyte proliferation (blastic transformation) in mice treated with epigallocatechin gallate alone or in combinations with any of the carcinogens (AFB_1, IQ or MNU) differed in no instance from controls. Bub *et al.* (2003) also described an increased lymphocyte proliferative responsiveness by epigallocatechin gallate. EGCG eliminated significantly the suppression of blastic transformation induced by all carcinogens tested.

The reparatory effect of epigallocatechin gallate on immunosuppression induced by all the carcinogens was demonstrated in the lethal phase of murine granulocytes, as well as during the functional activity of murine splenocytes. At the same time, the action of epigallocatechin gallate alone had a significant modulating character in natural as well as in adaptive immunity.

We can say that EGCG exerts, a wide variety of protective effects, and from this point of view it affects all stages of the carcinogenic process, i.e. initiation, promotion, and progression. Therefore, it is one of the most effective chemoprotective compounds.

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