Epigallocatechin gallate, the main polyphenol in green tea, binds to the T-cell receptor, CD4: Potential for HIV-1 therapy

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Background: The green tea flavonoid, epigallocatechin gallate (EGCG), has been proposed to have an anti–HIV-1 effect by preventing the binding of HIV-1 glycoprotein (gp) 120 to the CD4 molecule on T cells.

Objective: To demonstrate that EGCG binds to the CD4 molecule at the gp120 attachment site and inhibits gp120 binding at physiologically relevant levels, thus establishing EGCG as a potential therapeutic treatment for HIV-1 infection.

Methods: Nuclear magnetic resonance spectroscopy was used to examine the binding of EGCG and control, (-)-catechin, to CD4-IgG$_2$ (PRO 542®). Gp120 binding to human CD4$^+$ T cells was analyzed by flow cytometry.

Results: Addition of CD4 to EGCG produced a linear decrease in nuclear magnetic resonance signal intensity from EGCG but not from the control, (-)-catechin. In saturation transfer difference experiments, addition of 5.8 μmol/L CD4 to 310 μmol/L EGCG produced strong saturation at the aromatic rings of EGCG, but identical concentrations of (-)-catechin produced much smaller effects, implying EGCG/CD4 binding strong enough to reduce gp120/CD4 binding substantially. Molecular modeling studies suggested a binding site for EGCG in the D1 domain of CD4, the pocket that binds gp120. Physiologically relevant concentrations of EGCG (0.2 μmol/L) inhibited binding of gp120 to isolated human CD4$^+$ T cells.

Conclusion: We have demonstrated clear evidence of high-affinity binding of EGCG to the CD4 molecule with a K_d of approximately 10 nmol/L and inhibition of gp120 binding to human CD4$^+$ T cells.

Clinical implications: Epigallocatechin gallate has potential use as adjunctive therapy in HIV-1 infection. (J Allergy Clin Immunol 2006;118:1369-74.)

Key words: *HIV-1, gp120, CD4, EGCG, NMR, STD, flow cytometry*

Abbreviations used
EGCG: Epigallocatechin gallate
FITC: Fluorescein isothiocyanate
Gp: Glycoprotein
NMR: Nuclear magnetic resonance
STD: Saturation transfer difference

CD4 is a cell surface glycoprotein expressed on T cells and plays an important role in the recognition of antigens by T cells and in their activation.[1] It also acts as a receptor for HIV-1, because the viral envelope protein glycoprotein (gp) 120 binds to it via its D1 domain and uses this interaction to infect CD4$^+$ T cells.[1] Therefore, there has been interest in finding molecules that block the binding of gp120 to CD4 (entry inhibitors) as a way of reducing HIV-1 infectivity.[2]

Such a potential viral entry inhibitor is EGCG, a polyphenolic catechin that is one of the main active components of green tea. Among the properties ascribed to EGCG are antitumorigenic, anti-inflammatory, antioxidative, antiproliferative, antibacterial, and antiviral effects.[3-5] It is widely cited as a beneficial compound because of its ability to bind to a variety of other molecules via its polyphenolic rings.[6-10] It has, therefore, been suggested to have beneficial effects in a variety of diseases, particularly cancer,[11] but also HIV-1 infection.[4,5,12-14] Several mechanisms of the anti-HIV effects of EGCG have been suggested, and indeed it has been suggested that the anti-HIV effects of EGCG could arise from several factors acting in synergy.[4] EGCG destabilizes viral particles.[4] It inhibits HIV-1 replication in human PBMCs *in vitro* by inhibiting the biochemical activity of HIV-1 reverse transcriptase, the result being a subsequent decrease in HIV-1 p24 antigen concentration.[12]

An inhibitory effect on HIV replication is evident at concentrations down to 0.1 μmol/L.[5] At high concentrations (ie, nonphysiologic; 50-200 μmol/L), EGCG has been shown to prevent the attachment of HIV-1–gp120 to CD4 molecules on T$_H$ cells.[13] Also, recently it has been discovered that EGCG, at the nonphysiologic concentration of 200 μmol/L, blocked formation of the HIV-1 fusion-active core conformation, gp41 6-helix bundle.[14]

Here we study the interaction between EGCG and CD4, and present evidence that EGCG has the potential to exert

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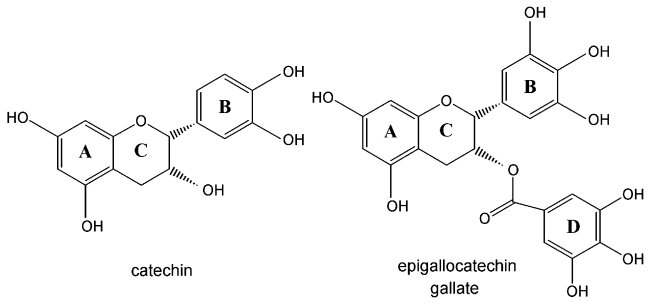

FIG 1. The structure of (-)-catechin and EGCG. The rings are labeled.

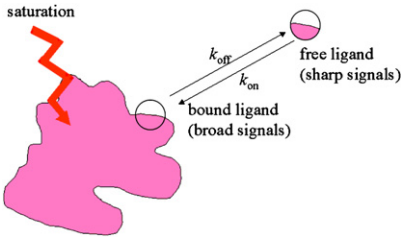

FIG 2. Transfer of saturation in NMR. Saturation of the protein by a radio frequency pulse *(red)* is transferred around the protein *(pink)* and onto the bound ligand. Exchange permits the saturation to be visible on the free ligand, thus identifying that the ligand binds and characterizing its binding site.

a protective effect *in vivo*, using physiologically attainable concentrations.[15]

METHODS

Reagents

Epigallocatechin gallate and (-)-catechin, a control polyphenol (Fig 1) that does not bind to CD4,[13] were gifts from Unilever Research, Colworth, United Kingdom. The CD4-IgG2 fusion protein, PRO 542, was provided gratis by Progenics Inc (Tarrytown, NY). PRO 542 is a novel inhibitor of HIV-1 attachment and entry. The soluble CD4 D1 domain, PRO 542, was used as a fusion protein of the D1D2 domains with the κ constant region of IgG_2 constant region, with 4 D1D2 domains on each construct in the positions of the light and heavy chain variable regions.[16]

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy was used because of its ability to yield high-resolution structural information regarding the binding interactions between ligands. Specifically, NMR characterizes structural processes that are dynamic (such as protein folding and binding events) and can provide information on binding affinities and locations (Fig 2).[17] NMR spectra of free ligand are sharp, but spectra of bound ligand are often so broad as to be invisible. When ligand is exchanging between free and bound, the signal seen depends on the off and on rates, and therefore on concentrations of ligand and protein and on K_d, thus permitting estimation of rates and affinities. This methodology has been employed in previous studies by Charlton et al.[10,18] Experiments were performed by using Advance 500, 600, and 900 spectrometers (Bruker, Germany) equipped with cryoprobes. Frequencies (chemical shifts) were measured in parts per million relative to the reference compound trimethylsilyl propionate at 0 ppm. The water signal was suppressed using a WATERGATE pulse sequence program before detection.

Saturation transfer difference

Saturation transfer difference (STD) is an NMR technique related to the transferred nuclear Overhauser effect that provides information on ligand binding to receptors, and in favorable cases can show which parts of the ligand are in contact with the receptor (Fig 2).[19,20] Saturation of signals of the receptor protein by a radiofrequency pulse is transferred to the parts of a bound ligand that are in contact with the protein, and from there to free ligand. In this way, reduction in signal intensity of the ligand (usually observed as a difference signal) indicates regions of the ligand in contact with the protein.[21] For the STD experiments, saturation alternated between 0.5 ppm (on resonance) and –10 ppm (off resonance), with typically 40 cycles of 16 scans at each frequency, and a 2-second saturation period, giving a total experimental time of approximately 45 minutes per experiment. All experiments were repeated to check for consistency of results.

Isolation of human peripheral blood $CD4^+$ T cells

$CD4^+$ T cells were positively selected from platelet-depleted human leukopaks to obtain a highly purified $CD4^+$ T-cell population. Briefly, CD4-coated magnetic beads (Dynal, Oslo, Norway) were added to the blood in a target-to-bead ratio of 1:5. The mixture was incubated at 4°C. The bead-cell complexes were collected and washed, and the cells were then separated from the magnetic beads. The assessment of the purity of the isolated cells was made by flow-cytometric measurement of CD3 (T-cell marker), CD4 (T_H cell receptor; 98% $CD4^+$), CD14 (monocyte receptor), CD20 (B-cell receptor), and CD45 (lymphocyte marker).

Human studies issues

Informed consent for HIV-1–negative donor blood was obtained and donor selection was made according to the Guidelines of the Gulf Coast Regional Blood Bank (Houston, Tex) in a manner approved by the Institutional Review Board at Baylor College of Medicine (H16902).

Flow-cytometric analysis

$CD4^+$ T cells were incubated for 1 hour at 37°C with differing concentrations of EGCG or (-)-catechin in RPMI-1640 supplemented with penicillin, streptomycin, glutamine, and heat-inactivated 2% FBS. After the incubation, cells were washed with media and incubated with 1 μg/mL fluorescein isothiocyanate (FITC)–conjugated recombinant gp120 (Immunodiagnostics, Woburn, Mass) for 30 minutes at room temperature. The fluorescent intensity of gp120-FITC

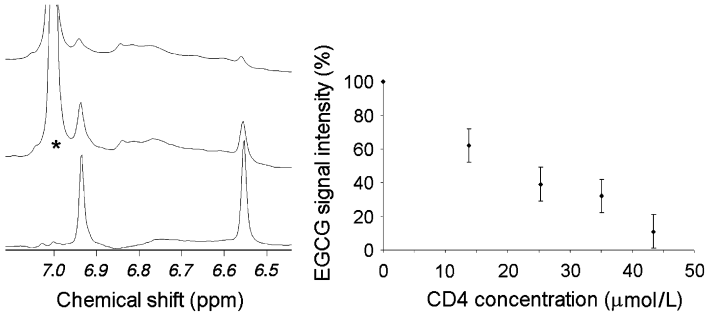

FIG 3. Loss of intensity of EGCG signals on titration with CD4. The spectra on the *left* show signals from rings D and B of EGCG (at 6.92 and 6.55 ppm respectively), with addition of CD4 domain *(from bottom to top)*. The signal marked with an *asterisk* is a low-molecular-weight compound present in the CD4 buffer. The EGCG signals decrease in intensity on addition of CD4, without any noticeable increase in line width. The graph on the *right* shows the approximately linear loss in intensity with increasing CD4 concentration.

bound to the surface of lymphocytes was measured with EPICS XL (Coulter, Hialeah, Fla).

Statistical analysis

Flow cytometry. Data generated from the flow cytometer were recorded and statistically analyzed using Coulter software. Calculation of fluorescence (expressed as median value of fluorescence emission curve) was conducted after conversion of logarithmically amplified signals into values on a linear scale. The statistical significance was calculated by using the parametric Kolmogorov-Smirnov test. Statistical significance was also determined by using the Student t test or 1-way ANOVA using SigmaStat software (Systat, Point Richmond, Calif). Values are expressed as means ± SEs of replicate assays. In all tests, $P < .05$ was considered statistically significant.

RESULTS

NMR titrations

A solution of 50 μmol/L EGCG was prepared in 50 mmol/L phosphate, pH 7.5. On titration of CD4 D1 domain in the same buffer, the NMR signal from EGCG disappeared in an approximately linear manner with concentration of CD4, without any evidence of exchange broadening (Fig 3). Loss of signal was not observed for (-)-catechin plus CD4 at identical concentrations, nor for EGCG plus a control IgG antibody not containing the CD4 domains (data not shown). The data imply binding of EGCG to CD4, which is tight enough to lead to essentially 100% binding at a concentration of 100 μmol/L, and significantly tighter than binding to control antibody. Using the conservative assumption of at least 90% of EGCG bound to protein, this implies a dissociation constant stronger than 1 μmol/L, probably at least 10 times stronger. The binding is indicated as being specific in that the control catechin or antibodies bound much more weakly.

STD

Fig 4 shows NMR and STD experiments of 1.45 μmol/L protein (5.8 μmol/L binding site) and 310 μmol/L EGCG, a ratio of EGCG to binding site of 53. Strong saturation was seen at the 4 signals arising from the aromatic rings of EGCG, indicating binding of EGCG to the protein at the polyphenolic rings (Fig 4, *A and B*). STD experiments performed at several different protein (PRO 542): ligand (EGCG) concentrations and ratios resulted in similar findings. STD experiments using a 1:1 ratio of EGCG to (-)-catechin (Fig 4, *C*) produced much larger effects on the EGCG than on catechin, and only a slight reduction in the STD on EGCG (Fig 4, *D*), suggesting that although there is some competition between (-)-catechin and EGCG, control catechin binds much more weakly than EGCG. Other low-molecular-weight components of the protein buffer (indicated by *asterisks* in Fig 4) produced no STDs, showing a lack of binding and therefore specific binding by the polyphenols. Finally, control experiments using a random IgG showed only weak effects to EGCG, implying some (but weaker) binding of EGCG to IgG (data not shown).

Modeling

In HIV-1 infection, interatomic contacts are made between 22 CD4 residues and 26 gp120 amino acid residues. The most critical of the CD4 residues are Phe 43 and Arg 59, with Phe 43 at the center of the cluster of residues involved in binding. Sixty-three percent of all interatomic contacts come from 1 span (40-48) in C′C′′ of CD4; Phe 43 alone accounts for 23% of the total.[22] Molecular modeling using the crystal structure coordinates 1CDJ[22] (Fig 5) suggests that there is an appropriate binding site for EGCG in the region of Phe 43, Arg 59, and Trp 62, which is the region of CD4 that interacts with gp120,[22,23] and would therefore prevent docking of gp120 onto the D1 domain.[24] EGCG is known to be particularly good at binding to arginine and aromatic residues, using mainly rings D and B.[10,18] The model therefore agrees well with known affinities of EGCG and with the measured STD effects. Modeling of interactions of CD4 with EGCG and gp120 has been performed independently[25] and agrees with the main features of our model, namely stacking of the galloyl ring D against Trp 62, and interactions with Arg 59 and Phe 43. This modeling study also concludes that the binding of EGCG to CD4 completely blocks binding of gp120. It therefore provides

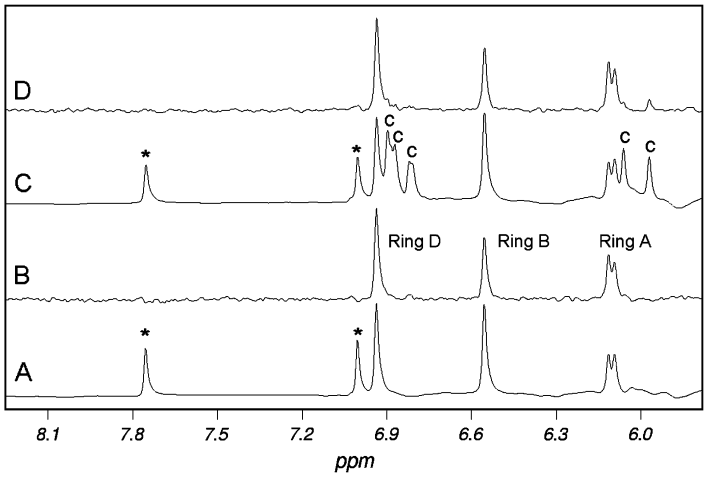

FIG 4. Saturation transfer difference spectra. **A,** NMR spectrum of 310 μmol/L EGCG in the presence of 1.45 μmol/L PRO 542 (5.8 μmol/L CD4). The signals marked with *asterisks* are from low-molecular-weight compounds present in the CD4 buffer. **B,** STD spectrum from this solution. The intensities of the STDs at rings D, B, and A are 10%, 7.4%, and 10%, respectively. **C,** NMR spectrum of 310 μmol/L EGCG plus 310 μmol/L (-)-catechin in the presence of 1.45 μmol/L PRO 542 (5.8 μmol/L CD4). The catechin signals are marked *c*. **D,** STD spectrum from this solution. The intensities of the STDs at rings D, B, and A of EGCG are 9%, 6%, and 9%, respectively, whereas the STDs from catechin are in the range 1.5% to 3%. In *B* and *D*, there are no measurable STDs to the other signals marked with asterisks.

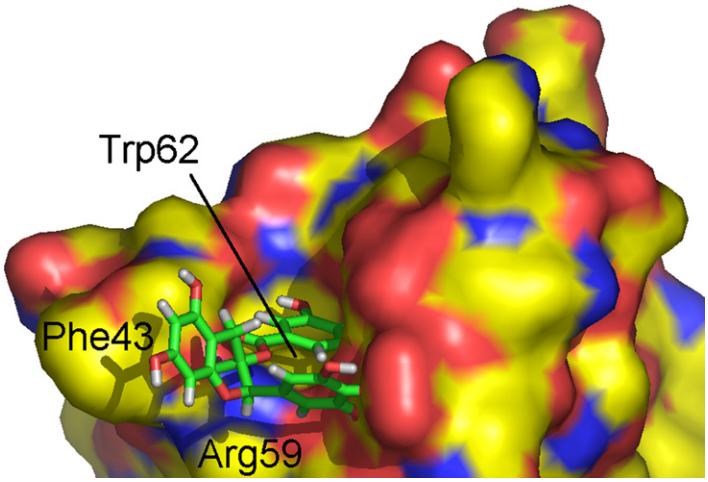

FIG 5. A model of EGCG binding to the D1 domain of CD4. Model drawn using PyMol, with the crystal structure coordinates 1CDJ.[22] In the protein, carbon is *yellow*, oxygen is *red*, and nitrogen is *purple*. The view is approximately from the direction of binding of gp120.

strong support of our work, both in the specificity of the interaction and in its consequences.

Inhibition of gp120 binding to CD4$^+$ T cells by EGCG

Determination of an inhibitory effect of gp120 binding on CD4$^+$ T cells by EGCG was made by analysis of the binding affinity of FITC-conjugated recombinant gp120 to the EGCG-treated and untreated CD4$^+$ T cells. Isolated CD4$^+$ T cells were treated for 1 hour with various concentrations of EGCG or the control polyphenol, (-)-catechin. EGCG significantly inhibited the binding of gp120 to CD4$^+$ T cells in a dose-dependent manner at 0.2 (42%), 2.0 (47%), and 20 μmol/L (55%) EGCG (P = .02, .006, and .001, respectively; Fig 6). Incubation with control, (-)-catechin, did not alter the binding capacity of gp120 on CD4$^+$ T cells (Fig 6). There was no statistical difference found in the EGCG-induced inhibition of gp120 binding to CD4$^+$ T cells whether the EGCG was washed out or remained after the incubation period (data not shown).

A major concern in assessing the therapeutic nature of EGCG as an inhibitor of HIV-1–gp120 binding to CD4$^+$ T cells is its potential to bind to serum proteins, resulting

in the alterations of its effectiveness as a blocker. In our experimental design, substitution of human serum for FBS resulted in confirmation of the inhibition of gp120 binding to $CD4^+$ T cells in the presence of EGCG in a dose-dependent manner at 0.2 (38%), 2.0 (42%), and 20 μmol/L (51%; $P < .01$).

DISCUSSION

Binding affinity of EGCG for CD4

The linear decrease of signal of free EGCG on titration with CD4, as well as the lack of exchange broadening, imply a dissociation constant stronger than 1 μmol/L, as described. On the other hand, the fact that an STD effect can be seen must imply an off-rate that is comparable to or faster than the cross-relaxation rate between CD4 and EGCG. This implies an upper limit to the dissociation constant of approximately 1 nmol/L. The dissociation constant, therefore, lies between 1 nmol/L and 1 μmol/L, and probably (for the reasons given) nearer to 1 nmol/L. We have therefore assumed an approximate K_d of 10 nmol/L.

Inhibition of gp120 binding on $CD4^+$ T cells by EGCG

Interference of gp120 binding to $CD4^+$ T cells was assessed at the physiologically relevant level of 0.2 μmol/L on the basis of evidence from the literature of plasma EGCG levels after 1 hour of ingesting green tea ranging from 0.1 to 0.6 μmol/L.[3,26] Under these conditions, we observed 40% inhibition of HIV-1–gp120 binding to $CD4^+$ T cells (Fig 6). Higher (nonphysiological) concentrations of EGCG were incapable of complete inhibition of this binding. Importantly, the inhibition of binding is dose-dependent.

Implications for competition with gp120 binding *in vivo*

It is possible to write a simple set of equations describing the competition between EGCG and gp120 for binding to CD4 in plasma. Writing C for CD4, E for EGCG and P for gp120,

$$[C] + [CE] + [CP] = [C]_0$$

$$[E] + [CE] = [E]_0$$

$$[P] + [CP] = [P]_0$$

where $[C]_0$ is the total concentration of CD4, $[E]_0$ is the total concentration of EGCG, and $[P]_0$ is the total concentration of gp120. An equation for the binding equilibrium of CD4 to EGCG can also be written.

The dissociation constant estimated in this study for the binding of CD4 to EGCG is 10 nmol/L, whereas for CD4 binding to gp120, the affinity has previously been calculated to be approximately 5 nmol/L.[27,28] In addition, we

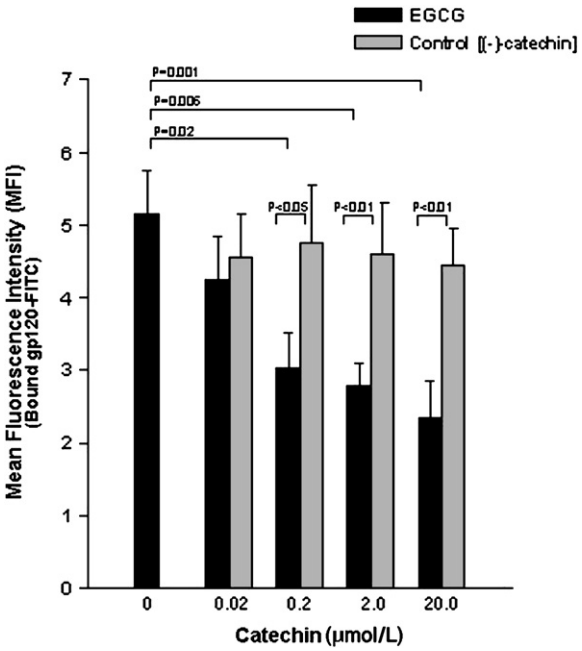

FIG 6. EGCG inhibition of gp120 binding to human $CD4^+$ T cells as assessed by flow cytometry. The data are expressed as means ± SDs of 6 independent experiments. Only significant differences are noted.

assume that there are approximately 500 T cells/μL of peripheral blood, each containing 50,000 $CD4^+$ molecules of CD4 per cell, and that during HIV-1 infection, there are 50,000 virus particles per milliliter, with 200 molecules of gp120 per virus. However, the gp120 molecules are grouped together, implying that there may be approximately 70 clusters of gp120 trimers per virus.[29,30] Finally, on the basis of the literature, we assume the concentration range of EGCG likely in the plasma after consuming the equivalent of 2 to 3 cups of green tea is within the range of 0.1 to 0.6 μmol/L and with greater consumption of green tea (7-9 cups) at the level of 1 μmol/L.[31-33]

The combination of these equations implies that in the absence of EGCG, approximately 0.8% of the gp120 clusters will be bound to CD4. However, in the presence of EGCG, the fraction bound is reduced to only 0.05%, resulting in a reduction of 16-fold. Reductions of approximately this magnitude are obtained using a wide range of estimates for these numbers. This of course does not provide complete inhibition of binding. Although this is probably not ideal (though Lipton[34] suggests that complete inhibition is often counterproductive), it is likely to provide a significant reduction in infectivity, and therefore a benefit to the patient.

In this study, we have not considered other possible binding sites for EGCG, so *in vivo* the effect is likely to be less than this. Nevertheless, the results suggest that EGCG, possibly given as a therapeutic intervention such as in a capsular form as an alternative to drinking green tea, could be a useful way of reducing the risk of HIV-1 infection.

We therefore conclude that EGCG at concentrations equivalent to those obtained by the consumption of green tea is able to reduce the attachment of gp120 to CD4 (when present at physiological concentrations) by a factor of between 10-fold and 20-fold. Although we would not advocate green tea as the sole prophylactic, it may be useful in combination with other antiretroviral therapies, and it provides a safe and enjoyable way of improving health generally.[35,36]

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