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Tea Pigments Inhibit the Production of Type 1 (T_{H1}) and Type 2 (T_{H2}) Helper T Cell Cytokines in $CD4^+$ T Cells

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Tea pigments are oxidized products of polyphenols derived from tea leaves (*Camellia sinensis*). Theaflavins are constituents of tea pigments with antioxidant, antineoplastic and antiinflammatory properties similar to their parent compounds. The biological properties of polyphenols and theaflavins have been linked to their capacity to inhibit the activation of nuclear factor-κB (NF-κB), a transcription factor, which is critically involved in the molecular regulation of a number of proinflammatory cytokines. The current study examines the requirement for NF-κB in the immunosuppressive effects mediated by tea antioxidants. Specifically, we tested the hypothesis that cytokines produced by type 1 (T_{H1}) $CD4^+$ T cells which require NF-κB for gene expression, such as interleukin-2 (IL-2) and interferon gamma (IFN γ), are selectively inhibited by tea pigments. We found that tea pigments potently suppress IL-2 secretion, IL-2 gene expression and the activation of NF-κB in murine spleens enriched for $CD4^+$ T cells, as expected. Consistent with our hypothesis, tea pigments also inhibited the induction of IFNγ mRNA. However, the expression of the T_{H2} cytokines IL-4 and IL-5, which lack functional NF-κB sites within their promoters was unexpectedly suppressed by tea pigments, as well. The results indicate that NF-κB may be only one of multiple transcription factors inhibited by tea pigments. Copyright © 2002 John Wiley and Sons, Ltd.

Keywords: tea pigments; theaflavins; NFκB; $CD4^+$ T cells; cytokines.

Introduction

Complementary approaches to traditional medicine, such as the use of antioxidants derived from foods offer great promise for the treatment of human diseases, in part because of their favourable toxicity profile. Polyphenolic components of higher plants, such as those present in green tea have been shown to exhibit cardioprotective and anticarcinogenic effects (Renaud and De Lorgeril, 1992; Ahmad and Mukhtar, 1999). Tea polyphenols are unstable and may be further oxidized to tea pigments. The theaflavins, theaflavin-3-gallate, theaflavin-3′-gallate, theaflavin-3,3′-digallate and theaflavin itself are major constituents of tea pigments with antioxidant, antimutagenic, antiproliferative and antineoplastic activities (Shiraki *et al.*, 1994) and account for the characteristic colour and taste of black tea.

A considerable literature suggests that polyphenols and theaflavins have antiinflammatory properties. Polyphenols in chocolate inhibited both mitogen-stimulated proliferation of peripheral blood T lymphocytes and polyclonal immunoglobulin production (Sanbongi *et al.*, 1997). Theaflavin abrogated lipopolysaccharide (LPS)-induced nitric oxide synthase (NOS) expression in murine macrophages (Lin *et al.*, 1999). The antioxidant properties of polyphenolic phytochemicals appear to contribute significantly to both their chemopreventive effects and their capacity to suppress inflammation. For example, catechins from green tea extract, and curcumin from turmeric are potent scavengers of oxygen and lipid radicals and have been shown to inhibit the development of both 12-0-tetradecanoyl-phorbol-13-acetate (PMA) induced skin cancer (Katiyar *et al.*, 1992) and skin inflammation in the mouse (Huang *et al.*, 1988). Tumorigenesis has in turn been associated with chronic inflammation (Cordon-Cardo and Prives, 1999). The molecular bases for the antiinflammatory effects of tea polyphenols and tea pigments remain to be completely clarified, but have been linked to inhibition of nuclear factor-κB (NF-κB), a transcription factor which governs the regulation of a wide range of molecules, including cytokines, chemokines, adhesion molecules, inducible NOS, prostaglandins and major histocompatibility (MHC) class II antigens (reviewed in Barnes and Karin, 1997). Kumar *et al.* (1998) demonstrated that pretreatment of endothelial cells with curcumin abrogated tumour necrosis factor alpha (TNFα)-induced activation of NF-κB. Similar results were obtained by Singh and Aggarwal (1995) using myelomonoblastic leukaemia cells, and by Jobin *et al.* (1999) in intestinal epithelial cells. Recently, Yang *et al.* (1998) showed that the green tea polyphenol epigallocatechin gallate (EGCG) sup-

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pressed LPS-induced TNFα production in murine peritoneal macrophages (Mϕ) by inhibiting NF-κB activation, and Lin *et al.* (1999) demonstrated that theaflavin inhibited LPS-mediated transcription of NOS mRNA in Mϕ-like RAW 264.7 cells by down-regulating the activation of NFκB.

A number of cytokines derived from $CD4^+$ helper T lymphocytes which utilize NF-κB as a transcription factor play an important role in cellular immunity. Such cytokines are capable of upregulating the expression of adhesion molecules on endothelial cells, of orchestrating the production of acute phase reactants by hepatocytes, of enhancing the synthesis of eicosanoids by macrophages, and of augmenting the density of MHC proteins on antigen presenting cells which together may amplify and perpetuate inflammation (Kumar *et al.*, 1998).

The pattern of cytokine production from helper T cells divides them into distinct subsets (Mosmann and Coffman, 1989; Romagnani, 1994). Type 1 helper T cells (T_{H1} cells) produce interleukin-2 (IL-2) and interferon gamma (IFNγ) after antigen receptor mediated stimulation, and orchestrate delayed-type hypersensitivity. T_{H2} cells coordinately produce IL-4, IL-5, and IL-6 and regulate humoral immunity. The T_{H1} cytokines IL-2 and IFNγ are key participants in the pathogenesis of inflammation (Romagnani, 1994), and immunotherapy directed against cytokines has been used in the treatment of human and experimental inflammatory diseases. Optimal transcription of T_{H1} cytokines, such as IL-2 and IFNγ is highly dependent upon NF-κB (Beauparlant and Hiscott, 1996). In contrast, transcription of T_{H2} cytokines, such as IL-4 and IL-5 (Lee *et al.*, 1994) does not require NF-κB. To better define the role played by NFκB in the immunosuppression mediated by tea antioxidants, we tested the hypothesis that tea pigments selectively inhibit gene expression of T_{H1} cytokines. Such a finding would provide a rational basis for the use of polyphenols and their stable oxidation products, tea pigments, in the treatment of inflammatory processes that are driven by aberrations in cell-mediated immunity. We report here that tea pigments potently suppress mitogen-induced mRNA expression of NF-κB-dependent T_{H1} family cytokines and nuclear translocation of NF-κB, as anticipated. However, the mitogen-stimulated gene expression of the T_{H2} cytokines, IL-4 and IL-5, which lack canonical NF-κB sites in their promoters, was also profoundly reduced. The results indicate that tea antioxidants may be more promiscuous in their immunosuppressive effects than previously realized, and suggest the need for a more precise understanding of the molecular mechanisms which underlie the salutary effects of polyphenols and their derivatives on inflammation.

Materials and Methods

Materials. Concanavalin A (Con A), LPS (*E. coli* 055:B1), PMA, Taq DNA polymerase and trypan blue were purchased from Sigma Diagnostics (St Louis, MO). RPMI 1640 and Hanks balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY). Penicillin/streptomycin and glutamate were purchased from Quality Biological, Inc. (Gaithersburg, MD). dNTPs were obtained from Amersham (Arlington Heights, IL). Fetal calf serum (FCS) was purchased from Hyclone (Logan, UT). Recombinant murine IL-1β was obtained from R&D Systems, Inc. (Minneapolis, MN). [γ^{32P}]ATP was purchased from ICN (Costa Meda, CA). [α^{32P}]CTP was obtained from NEN (Boston, MA). Anti-p65 and anti-P50 antibodies were purchased from Santa Cruz (Santa Cruz, CA). Anti-CD8 antibody (clone 53-6.7) was obtained from Pharmingen (San Diego, CA). Rabbit complement was purchased from Cederlane laboratories (Ontario, Canada). Anti-mouse Ig was obtained from Biosource International (Camarillo, CA). The protein content of nuclear extracts was quantified using a kit from Biorad (Hercules, CA).

Preparation of tea pigments. Tea leaves were provided by Professor Junshi Chen, Institute of Nutrition and Food Hygiene, Chinese Academy of Preventive Medicine, Beijing, China. The preparation was prepared by extracting tea leaves with hot water. Aqueous extracts were then filtered, concentrated and extracted twice with chloroform to remove caffeine. The remaining aqueous phase was extracted twice with ethyl acetate to remove tea polyphenols and residual chloroform, then collected and air-dried under vacuum. The extract powder was subsequently dissolved in ethanol and filtered. Tea pigments were obtained after ethanol was evaporated under vacuum. Tea pigments, also referred to in this paper as TE, have a dark brown colour and are readily water soluble. The preparations used in our studies were devoid of caffeine and polyphenols and contained approximately 10% theaflavins.

Mice. Female and male CBA/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used at 6–8 weeks of age. Animals were cared for in accord with Institutional Animal Care and Utilization Guidelines.

Cell culture. Unfractionated spleen cells (5×10^6/mL), were prepared as previously described (Tomita-Yamaguchi *et al.*, 1991). CD4-enriched populations (hereafter referred to as $CD4^+$ T cells) were prepared by negative selection, sequentially depleting B cells and then $CD8^+$ T cells. B cells were depleted by suspending spleen cells (10^7/mL) in media consisting of HBSS plus 0.02% NaN_3 and 5% FCS, and incubating the mixture on 100 mm plastic petri dishes precoated with 5 μg of anti-mouse Ig at 4 °C for 70 min, as earlier reported (Tomita-Yamaguchi *et al.*, 1991). The nonadherent cells were recovered, washed in RPMI-1640 plus 5% FCS, and the procedure was repeated. $CD8^+$ T cells were removed by incubating B cell-depleted preparations with anti-CD8 antibody (clone 53-6.7) for 45 min at room temperature, then washing and incubating cells at 37 °C for 45 min with rabbit complement as previously described (Tomita-Yamaguchi *et al.*, 1991).

$CD4^+$ T cells were cultured in medium consisting of RPMI-1640 supplemented with penicillin (100 U/mL)/streptomycin (100 μg/mL), glutamate (2 mM) and 10% FCS in the presence or absence of varying doses (0.1–50 μg/mL) of Con A plus PMA (up to 100 ηg/mL), and varying concentrations of TE pigments (0–500 μg/mL) for up to 72 h. In assessing cytokine mRNA levels, cells were harvested after 4 h of culture. For quantification of IL-2 activity, supernatants were harvested after 16 h of culture. In order to evaluate the effect of tea pigments on

mitogen-stimulated proliferation, cells were analysed at 48 h. Nuclear extracts were prepared from $CD4^+$ T cells (5×10^6/mL) cultured for 15 min with or without Con A (1 μg/mL) plus PMA (20 ηg/mL) in the presence or absence of TE pigments (200 μg/mL). To examine the influence of IL-1 on tea-mediated inhibition of Con A-stimulated IL-2 production, cells (5×10^6/mL) were cultured with and without up to 650 ρg/mL of murine IL-1β in the presence of Con A (1μg/mL) plus PMA (20 ηg/mL) ± tea pigments (200 μg/mL). To determine the maximum IL-1β production from spleens, unfractionated spleen cells (5×10^6/mL) were cultured with LPS (up to 100 μg/mL) for 16 h.

RNA extraction and semi-quantitative RT-PCR analysis. RNA was isolated, reverse transcribed and amplified as previously described (Santoro *et al.*, 1995). PCR was performed on various amounts of 1:50 and 1:150 dilutions of the newly synthesized cDNA from each sample with β actin primers for standard curve titration using a modified protocol of Cai *et al.* (1993). Briefly, PCR was performed in a 25 μL reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 mg/mL BSA, 50 μM of each dNTP, 250 nM of β actin-specific primer and 1.0 units of Taq DNA polymerase. Following initial denaturation at 94 °C for 5 min, PCR was performed for 25 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 20 s, and primer extension at 72 °C for 1 min. There followed a final extension at 72 °C for 5 min in order to ensure completion of all reactions. PCR products were electrophoretically sized on 1.0% agarose gels, and negative images were taken with positive/negative film (Polaroid Corp., Cambridge, MA). Band intensities were obtained using a ScanJet 3c scanner (Hewlett Packard, MA), and the relative values were calculated using the NIH Image Analysis 1.58 computer program. Following normalization, PCR was performed using primers for IL-2, IL-4, IL-5, IFNγ and β actin purchased from Clontech (Palo Alto, CA). PCR products for IL-2, IL-4, IFNγ and B actin were electrophoresed on 1.0% agarose gels and visualized by ethidium bromide staining. In the case of IL-5, and for titration of IL-2 mRNA in response to Con A ± tea pigments, gels were subjected to Southern analyses as described earlier (Cai *et al.*, 1993). The IL-2, IL-5 and β actin probes were 30 mers obtained from Clontech.

Electrophoretic mobility shift assays. Nuclear extracts were prepared by a modification of the method by Franzoso *et al.* (1994). Briefly, cells were washed with a buffer (A) containing HEPES 10 mM, pH 7.3, KCl 15 mM, $MgCl_2$ 2 mM, EDTA 0.1 mM, DTT 1 mM, and PMSF 1 mM, then incubated with 0.2% NP40 in buffer A on ice for 1 min. The cells were then vortexed for 1 min and nuclei were recovered by centrifugation. Nuclear proteins were extracted and gel retardation assays were performed as previously described (Franzoso *et al.*, 1994) using a doublestranded, palindromic NF-κB oligonucleotide (5′GATCCCAACGGCAGGGGAATTCCCCTCTCCTTA-3′). Nuclear extracts containing 2 μg of protein were incubated with the end-labelled, NF-κB probe for 30 min at room temperature in the presence of poly dIdC (Pharmacia, Piscataway, NJ). Reaction mixtures were run on a 4% polyacrylamide gel (Boehringer Manheim, Indianapolis, IN). Competition experiments were performed with a 20-fold excess of unlabelled oligonucleotide containing the relevant sequence. Supershifts were performed using an anti-p65 antibody as previously reported (Franzoso *et al.*, 1994).

IL-2 and IL-1 ELISAs. ELISA kits for murine IL-2 and IL-1β were purchased from Biosource International, and performed on triplicate samples of supernatants derived from cell cultures, according to the manufacturer's instructions.

Viability and proliferation of cells. Tea pigment toxicity was assessed on $CD4^+$ T cells cultured in the presence of medium only, Con A plus PMA, tea pigments, or Con A plus PMA plus tea pigments for up to 72 h. At varying times, cells were harvested and subjected to staining with trypan blue, and the supernatants were analysed for LDH activity using a kit (Sigma) in accordance with the manufacturer's recommendation. Cell growth was assessed using a non-radioactive cell proliferation assay (Promega, Madison, WI) as instructed by the manufacturer.

Results

Effect of tea pigments on IL-2 expression

This study examines the potential immunosuppressive effects of tea pigments on cytokine production by Type 1 (T_{H1}) and Type 2 (T_{H2}) helper T cells. Spleen cells, as opposed to cell lines, were chosen as the source of $CD4^+$ T cells in order to provide an immunologically natural target for tea pigments. We used the T cell mitogen, Con A, as the stimulant. Cells were co-cultured with PMA, which provides an IL-1-like signal to T cells to obviate a potential confounding effect of tea pigments on macrophage-mediated cytokine production. In preliminary experiments, we defined the optimal conditions for cellular proliferation and IL-2 production (Fig. 11). We found that maximum proliferation (Fig 1A. left panel) and IL-2 production (Fig. 1A, right panel) of $CD4^+$ T cells (5×10^6/mL) occurred with a dose of 0.5–1 μg/mL of Con A in the presence of 20 ηg/mL of PMA and optimal cell growth occurred after 48 h of culture (Fig. 1A, inset). When cells were cultured with Con A (1 μg/mL), concentrations of PMA greater than 20 ηg/mL did not enhance cellular proliferation (data not shown). Thus, subsequent experiments were carried out using Con A at a dose of 1μg/mL and PMA at a concentration of 20 ηg/mL.

When $CD4^+$ T cells were cultured with Con A in the presence of tea, both cellular proliferation (Fig. 1B, left panel) and IL-2 production (Fig. 1B, right panel) were, essentially completely abrogated at doses of 200 μg/mL of TE pigments. This dose of tea pigments contains a concentration of theaflavins (~25 μM), which inhibits PMA-induced cell transformation *in vitro* (Dong *et al.*, 1997). The inhibitory effect of tea pigments on mitogen-mediated activation of splenic $CD4^+$ T cell was not due to cellular toxicity, as evidenced by the fact that doses of up to 500 μg/mL of tea pigments did not alter viability by trypan blue staining, or induce LDH release into the supernant after up to 3 days of culture when compared with cells incubated with vehicle only.

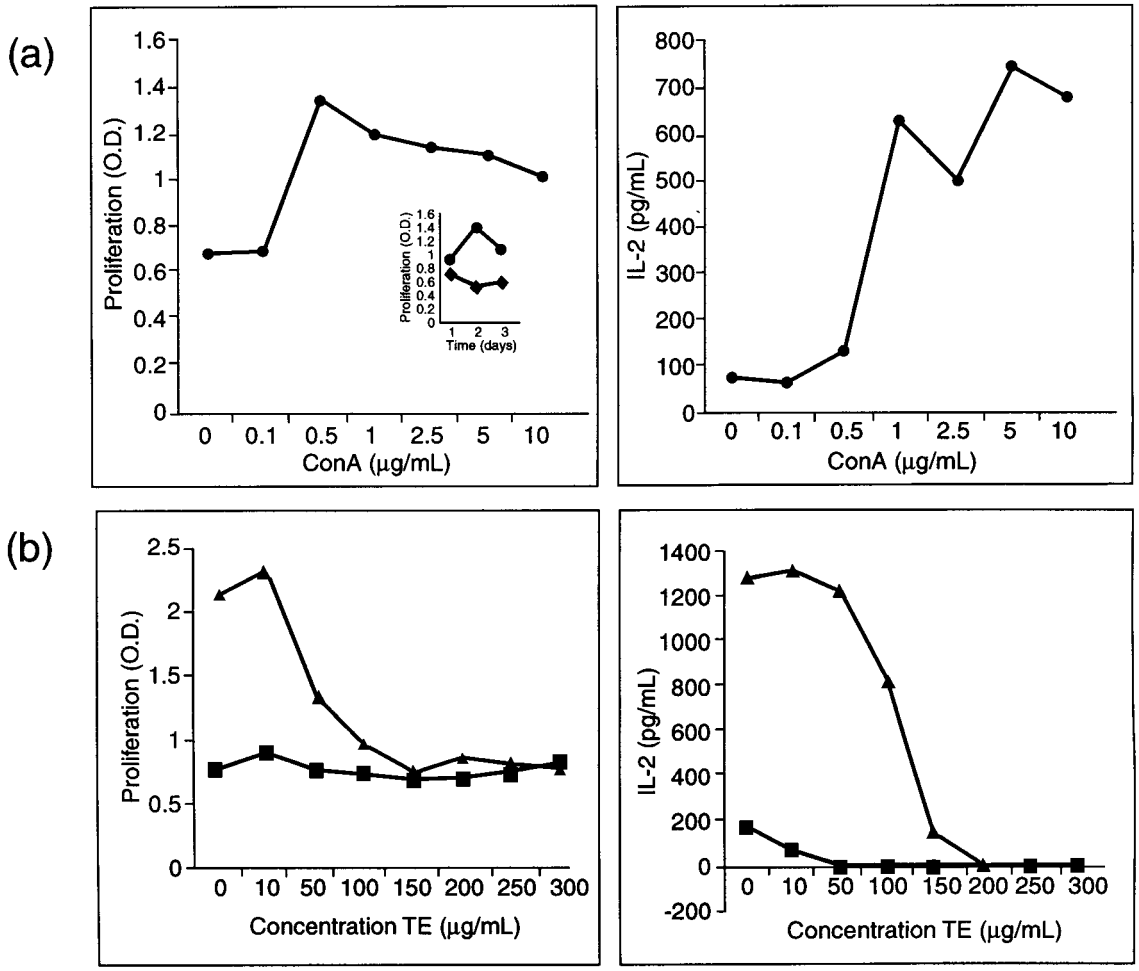

Figure 1. A. Proliferation and IL-2 production of $CD4^+$ T cells. $CD4^+$ T cells (5×10^6/mL) were cultured with various amounts of Con A for up to 72 h in the presence of PMA (20 ηg/mL). Left panel, cellular proliferation, determined at 48 h. Right panel, IL-2 production, measured at 16 h. Inset, kinetics of cellular proliferation of $CD4^+$ T cells (5×10^6/mL), examined at 24, 48 and 72 h, in response to Con A (1 μg/mL) plus PMA (20 εg/mL) (*circles*), or medium only (squares). B. Effect of TE pigments on proliferation and IL-2 production of $CD4^+$ T cells. $CD4^+$ T cells (5×10^6/mL) were cultured in medium only (*squares*), or with 1 μg/mL of Con A plus 20 ηg/mL of PMA (*triangles*) in the presence of various concentrations of TE pigments and cellular proliferation after 48 h (left panel), and IL-2 production after 16 h (right panel) of culture were determined.

T cell growth depends on IL-2 production. IL-2 synthesis, in turn requires IL-1β, a product of macrophages. As stated above, PMA was used in our culture system in order to bypass the IL-1 requirement of T cells. To address the unlikely possibility that IL-1β would provide a signal distinct from that of PMA, exogenous IL-1β was added to $CD4^+$ T cells cultured with mitogen plus tea pigments. The dose of IL-1β chosen was determined in experiments in which unfractionated spleen cells (5×10^6/mL) were cultured with the macrophage stimulant LPS (0.01–100μg/mL) for 16 h to produce maximal levels of IL-1β. Peak IL-1β production under these conditions was 650 ρg/mL. Culture of $CD4^+$ T cells (5×10^6/mL) with IL-1β (650 ρg/mL) plus Con A (1 μg/mL) and PMA (20 ηg/mL) for 16 h in the presence of tea pigments (200 μg/mL) resulted in 87% and 98% reductions in IL-2 production compared with cells cultured in the absence of tea pigments in two separate experiments. These data suggest that inhibition of IL-1β cannot explain the suppression of mitogen-stimulated IL-2 synthesis by tea pigments.

We next examined the capacity of tea pigments to suppress IL-2 mRNA expression. In preliminary experiments, we found that peak steady state IL-2 mRNA levels occurred after a 4 h culture of $CD4^+$ T cells (5×10^6/mL) with Con A (1 μg/mL) plus PMA (20 ηg/mL) and returned to baseline by approximately 20 h (not shown). These results are in agreement with those of others (Jain *et al.*, 1995). In the presence of tea pigments, mitogen-stimulated IL-2 mRNA expression declined in a dose-dependent fashion (Fig. 2). The potency of tea pigment in suppressing steady state IL-2 mRNA expression was similar to that observed for inhibition of IL-2 protein

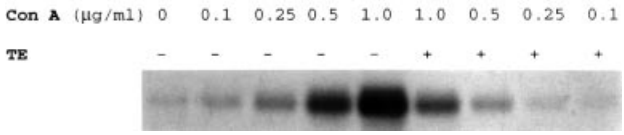

Figure 2. Effect of TE pigments on IL-2 mRNA expression. $CD4^+$ T cells were cultured for 4 h with Con A (1 μg/mL) plus PMA (20 ηg/mL) in the presence or absence of various amounts of TE pigments, mRNA was then isolated, reverse transcribed, and amplified. PCR products were run on a 1% agarose gel, and subsequently hybridized with an IL-2 probe.

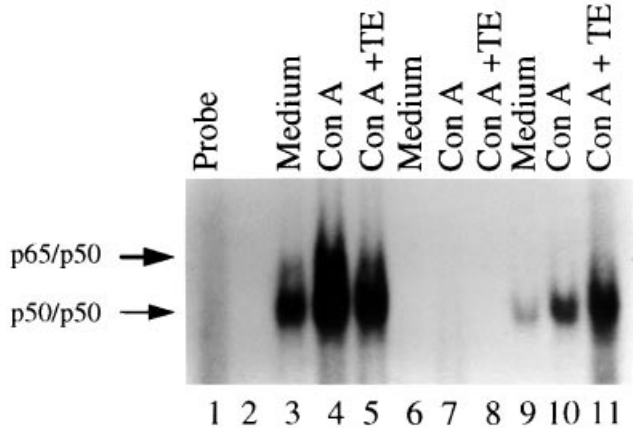

Figure 3. Effect of TE pigments on nuclear translocation of NF-κB. CD4$^+$ T cells (5×10^6/mL) were cultured for 15 min with or without Con A (1 μg/mL) plus PMA (20 ηg/mL) in the presence or absence of TE pigments (200 μg/mL), and nuclear extracts were prepared. Nuclear protein was then incubated with an end-labelled, double-stranded palindromic NF-κB oligonucleotide in the presence of poly dIdC. The reaction mixture was then resolved on a 4% polyacrylamide gel. Lane 1, probe only (the probe was run off the gel and is not shown). Lanes 3, 6, 9, cells cultured in medium only. Lanes 4, 7, 10, cells cultured with Con A (plus PMA) only. Lanes 5, 8, 11, cells cultured with Con A (plus PMA) plus TE pigments. Lanes 3–5, nuclear extracts incubated with labelled probe only. Lanes 6–8, nuclear extracts were incubated with a 20-fold excess of cold NF-κB probe. Lanes 9–11, nuclear extracts were preincubated with anti-p65 antibody. The supershifted complex migrated well above the region of the gel depicted, and is not shown.

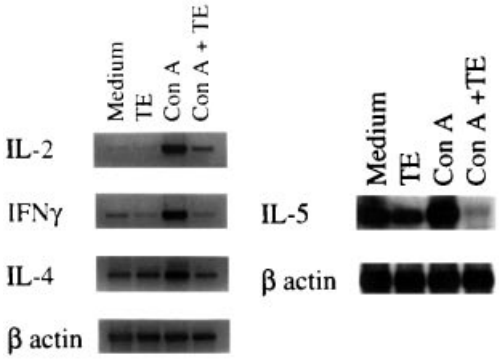

Figure 4. Effect of TE pigments on IFNγ, IL-4 and IL-5 expression. CD4$^+$ T cells (5×10^6/mL) were cultured in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of Con A (1 μg/mL) plus PMA (20 ηg/mL) and/or TE pigments (200 μg/mL) (lanes 2 and 4) for 4 h. Left panel, mRNA was isolated, reverse transcribed, and amplified using primers for IL-2 (upper series), IFNγ (second from top), IL-4 (third from top) and β actin (lowest series). PCR products were visualized by ethidium bromide staining. Right panel, effect of TE pigments on IL-5 gene expression. CD4$^+$ T cells (5×10^6/mL) were cultured for 4 h without (lanes 1 and 2), or with (lanes 3 and 4) Con A (1 μg/mL) plus PMA (20 ηg/mL) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of TE pigments (200 μg/mL). mRNA was isolated, reverse transcribed, and amplified using primers for IL-5 (upper) and β actin (lower). PCR products were run on a 1% agarose gel, and subsequently hybridized with IL-5 or β actin probes.

synthesis. The results suggest that tea pigments abrogate CD4$^+$ T cell IL-2 production at a transcriptional, or (less likely) posttranscriptional level.

Effect of tea pigments on NF κB

The effect of tea pigments (200 μg/mL) on the nuclear translocation of NF-κB in CD4$^+$ T cells (5×10^6/mL) stimulated with Con A (1 μg/mL) plus PMA (20 ηg/mL) was next investigated. As anticipated, tea pigments significantly inhibited the binding activity of NF-κB to the κB probe (Fig. 3). The inhibitory effect of tea pigments was specific, binding of the nuclear extract was abrogated by preincubation with a cold NFκB probe, and involved a failure to translocate the p65/p50 heterodimer, as shown by supershift experiments using anti-p65 antibody (Fig. 3).

Influence of tea pigments on cytokine gene expression of T_{H1} and T_{H2} cells

We investigated the influence of tea pigments on the expression of other cytokine genes. IFNγ is produced by T_{H1} cells. Its transcription parallels that of other members (e.g. IL-2) of a cytokine gene family whose promoter activity is regulated by NF-κB (Jain *et al.*, 1995). Co-culture of CD4$^+$ T cells (5×10^6/mL) with Con A (1 μg/mL) plus PMA (20 ηg/mL) and tea pigments (200 μg/mL) for 4 h significantly decreased mitogen-induced IFNγ mRNA expression as well as that of IL-2 mRNA (Fig. 4, left panel).

To determine whether CD4$^+$ T cells which produce T_{H2} cytokines, and lack canonical NF-κB binding sites in their promoter (Lee *et al.*, 1994), are susceptible to the inhibitory effects of TE, CD4$^+$ T cells (5×10^6/mL) were cultured with Con A (1 μg/mL) plus PMA (20 ηg/mL) in the presence of tea pigments (200 μg/mL) for 4 h and examined for IL-4 and IL-5 mRNA expression. The results demonstrated that gene expression of both IL-4 (Fig. 4, left panel) and IL-5(Fig. 4, right panel) was markedly downregulated by tea pigments.

Discussion

The rationale for this study derives from two divergent literatures. The first suggests that cytokines produced by T_{H1} cells, such as IL-2 and IFNγ, which orchestrate cell-mediated immunity, are proinflammatory and play a critical role in the pathogenesis of persistent inflammation (Romagnani, 1994). Chronic inflammation has, in turn, been linked to tumorigenesis (Cordon-Cardo and Prives, 1999). In contrast, cytokine products of T_{H2} cells, such as IL-4, have been labelled antiinflammatory and are typically reduced in inflammatory states characterized by cellular hyperreactivity (Romagnani, 1994). The second literature indicates that polyphenols, such as those contained in green tea extract (e.g. catechins), and in turmeric (e.g. curcumin), and derivatives of polyphenols (e.g. theaflavins) have potent antineoplastic and antiinflammatory properties (Ahmad and Mukhtar, 1999) and have the capacity to inhibit NF-κB, a ubiquitious protein involved in the genetic control of numerous regulatory molecules (Barnes and Karin, 1997; Beauparlant and Hiscott, 1996). Unlike the classical T_{H2} cytokines, IL-4 and IL-5, the proinflammatory cytokines IL-2 and IFNγ

contain functional NF-κB binding sites within their promoters. This formed the basis for our hypothesis that T_{H1} cytokines may be preferentially targeted by TE pigments, which are potent antioxidants and share a similar spectrum of biological activities with polyphenols of tea extract.

We found that TE pigments, containing concentrations of theaflavins which are known to be anticarcinogenic *in vitro* (Dong *et al.*, 1997), inhibit IL-2 protein secretion, IL-2 gene expression and NF-κB binding to its *cis*-acting element. The overall magnitude of potency suggested that the three events might be related. Consistent with our thesis, TE pigments also inhibited IFNγ gene expression, another cytokine produced by T_{H1} cells that is transcriptionally regulated by NFκB. However, our study revealed that TE pigments are also potent inhibitors of IL-4 and IL-5 gene expression. The promoters of these T_{H2} cytokines lack consensus NF-κB sites (Lee *et al.*, 1994). The results indicate that neither the pattern of cytokine secretion by $CD4^+$ T cells (i.e. T_{H1} versus T_{H2}), nor the presence of a functional NF-κB site predicts sensitivity to the immunosuppressive effects of TE pigments.

The molecular bases for our results remain speculative. Stimulation of $CD4^+$ T cells is accompanied by the activation of a number of transcription factors, such as NF-κB, nuclear factor of activated T cells (NF-AT) and nuclear factor P [NF(P)]. The coordinate activation of proteins such as these within a particular cell is thought to be required for maximum cytokine gene expression to be manifested. The P sequence of the IL-4 promoter (nucleotides -79 to -69, relative to the transcription initiation site) binds NF(P) and is a response element for Jurkat cells stimulated with calcium ionophore plus PMA (Szabo *et al.*, 1993). At least four P sequence-like elements are found in the 5′ upstream region of the mouse IL-4 gene and the binding of NF(P) to its *cis*-acting element appears to be required for induction of IL-4 gene expression (Szabo *et al.*, 1993). NF-AT is a large family of transcription factors comprising at least four members that are differentially expressed in lymphoid and nonlymphoid cells (Rao *et al.*, 1997). NF-AT is composed of the activator protein-1 (AP1) and non-AP1 proteins. The non-AP1 proteins undergo nuclear translocation following calcium influx during T cell activation. This event is blocked by cyclosporin A or FK506 (Flanagan *et al.*, 1991). NF-AT has been implicated in the transcriptional regulation of IL-2, IFNγ and IL-5. NF-AT binding sites have also been found in the IL-4 gene (Campbell *et al.*, 1996). Since the molecular mechanisms which operate in the antiinflammatory effects of tea pigments and their putative active ingredients, theaflavins, are not fully understood, it remains possible that tea pigments are capable of inhibiting transcription factors apart from NF-κB, such as NF-AT and/or NF(P). In support of this thesis is the report by Jobin *et al.* (1999) which indicates that the inhibitory effect of curcumin on TNFα-stimulated intestinal epithelial cells was not restricted to the NF-κB pathway, since the AP-1 pathway was also blocked. In fact, the data of Jobin *et al.* (1999) suggest that curcumin may act upstream of NF-κB-inducing kinase (NIK), an enzyme which participates in the activation of IκB kinase (IKK). It is the activation of IKK that results in the phosphorylation and subsequent ubiquination of IκB, leading to the release and nuclear translocation of NF-κB (Baeuerle and Henkel, 1994). Jobin *et al.* (1999) demonstrate that neither NIK, nor IKK, are directly inhibited by curcumin. IKK, and perhaps NIK, is a downstream target of mitogen activated protein kinase (MEKK), as is c-Jun N-terminal kinase (JNK). Chen and Tan (1998) have found that curcumin inhibits the JNK signalling pathway. Thus, MEKK may be the proximal target for curcumin, green tea polyphenols, and tea pigments.

The apparent lack of specificity of tea pigments for inhibition of cytokine gene expression in T_{H1} and T_{H2} cells has direct implications for clinical studies. Skewed T_{H1} and T_{H2} responses are a feature of a number of human and experimental disease states (Romagnani, 1994), including cancer (Sato *et al.*, 1998). The results of this study suggest that the possibility that a wide range of cytokines derived from $CD4^+$ T cells, and potentially a broad spectrum of immune activities may be targeted by tea pigments, and emphasize the need to more precisely define the immunosuppressive properties of this potentially highly useful therapeutic modality.

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