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Effect of *Theobroma cacao* flavonoids on immune activation of a lymphoid cell line

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We analysed the effect of (−)-epicatechin and cocoa extract on the activation of a lymphoid cell line. Particularly the expression of IL-2 receptor α (IL-2Rα or CD25) and, the secretion of IL-2 and IL-4 were established after flavonoid treatment. Two media culture conditions (1 and 10 % of fetal calf serum supplementation) and the different moments of flavonoid addition (simultaneously or 2 h before cell-activation) were compared. IL-2Rα (CD25) expression on activated cells was significantly reduced by epicatechin and cocoa extract in a dose-dependent manner, achieving the highest inhibition of about 50 % when flavonoids were added 2 h before stimulation. IL-2 secretion was also inhibited by the presence of both epicatechin and cocoa extract, displaying 60 and 75 % of inhibition, respectively. Cocoa flavonoids were also able to enhance 3–4·5-fold IL-4 release. In summary, cocoa extract down-modulated T lymphocyte activation and therefore the acquired immune response. This fact could be important in some states of the immune system hyperactivity such as autoimmune or chronic inflammatory diseases.

***Cocoa*: Epicatechin: Lymphocyte: IL-2**

At present, the apparent relationship between diet components and immune functions is clear (Kelly & Bendich, 1996; Kubena & McMurray, 1996; Field, 2000). Scientists have understood since the 1960s that the immune system plays a critical role in the relationship between malnutrition and infection. However, a major focus of current research is the possibility of modulating the immune system of healthy people by nutrition with the goal of improving health. For example, some scientists have shown that supplementation with certain nutrients, especially those with antioxidant activities, may improve immune function (Kelly & Bendich, 1996; Meydani *et al.* 1998).

Cocoa (*Theobroma cacao*) was considered a divine food by the Maya and Aztec civilisations; Mayans were consuming products derived from cocoa from as early as 600 BC (Hurst *et al.* 2002). Several documents, among them the Badianus Manuscript, the Florentine Codex and the Princeton Codex, show the use of cocoa for medicinal purposes. Over 100 different therapeutic uses are described: chocolate was eaten to treat heart pain, intestinal complaints and infections, and to reduce fatigue and increase sexual appetite (Dillinger *et al.* 2000).

Cocoa seeds are a rich source of flavonoids, mainly, (−)-epicatechin, (+)-catechin and polymers derived from these monomers called procyanidins (Hammerstone *et al.* 1999). The cocoa flavonoid content is difficult to establish because it depends on many factors (geography, climate, storage conditions, manufacturing processes, food matrix etc.; Manach *et al.* 2004). Cocoa powder is described as capable of reaching up to 70 mg/g polyphenols (expressed as catechin), whereas chocolate contains between 5 and 8·4 mg/g depending on the type (Vinson *et al.* 1999; Waterhouse *et al.* 1996). Quercetin, isoquercitin (quercetin-3-*O*-glucoside) and quercetin-3-*O*-arabinose are also present in cocoa powder in lower quantities although it is believed that these compounds might be important contributors to the health benefits of cocoa (Lamuela *et al.* 2001). Recently, other flavonoids have been identified in cocoa powder, such as hyperoside (quercetin-3-*O*-galactoside), naringenin, luteolin, apigenin and some O-glucosides and C-glucosides of these compounds (Sánchez-Rabaneda *et al.* 2003).

Little is known about the bioavailability of procyanidins. A recent human feeding study has proved that cocoa procyanidins are stable during gastric transit (Rios *et al.* 2002). Epicatechin and catechin are rapidly absorbed and found in human plasma (Richelle *et al.* 1999; Baba *et al.* 2000; Bell *et al.* 2000). In addition, Holt *et al.* (2002) have detected procyanidin dimer B2 (epicatechin-(4β-8)-epicatechin) in human plasma 30 min after cocoa beverage consumption.

There are some studies that show the contribution of cocoa and chocolate to the total flavonoid intake. Arts *et al.* (1999), in a Dutch nutritional survey, found that cocoa products contribute 20 % of the dietary catechins intake in this population. Moreover, according to the food consumption data of the Spanish Department of Agriculture, Fishery and Food (2002), cocoa products

Abbreviations: FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IL-2Rα or CD-25, interleukin-2 receptor α; mAb, monoclonal antibody; MFI, mean fluorescence intensities; PMA, phorbol 12-myristate 13-acetate; Th lymphocytes, T helper lymphocytes.

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can be an important source of dietary flavonoids in children and teenagers because cocoa powder consumption is relatively high, about 6–8 kg per child per year, equating to a consumption of about 20 g/d cocoa powder containing 5 g cocoa, 14 g sugar and 0·5 g fat. The effects that this cocoa consumption may represent have not yet been well established.

Cocoa flavonoids are antioxidants (Pietta, 2000). These flavonoids could be beneficial in some situations such as major chronic diseases with high presence of reactive oxidant species such as CHD (Weisburger, 2001; Steinberg *et al.* 2003; Kris-Etherton & Keen, 2002). It has also been reported that flavonol-rich cocoa inhibits epinephrine-stimulated platelet activation and function, qualitatively similar to aspirin (Pearson *et al.* 2002). The effects of cocoa flavonoids on the immune system are less known and are centred on the inflammatory response of macrophages and monocytes. Thus, cocoa polyphenols inhibit anion superoxide and hydrogen peroxide on activated granulocytes (Sanbongi *et al.* 1997). Moreover, cocoa procyanidins are able to decrease plasma leukotriene concentrations (Schramm *et al.* 2001). In addition, isolated procyanidin fractions have different effects on the secretion of certain cytokines (IL-1β, TNF-α, TGF-β_1, IL-2, IL-5 and IL-4) from peripheral mononuclear cells, depending on oligomer length (Mao *et al.* 2000*a*,*b*,*c*, 2002*a*,*b*, 2003). These suggestions prompted us to extend the research focused on the study of total polyphenols contained in cocoa powder on lymphocyte T activation. In the present work, we analyse the effect of (−)-epicatechin and cocoa extract on the expression of IL-2 receptor α (IL-2Rα or CD25) as well as on the secretion of IL-2 and IL-4 from stimulated murine EL4.BU.OU6 cell line. IL-2 and IL-2Rα are markers of early lymphocyte T activation that are involved in both humoral and cellular specific immune response. IL-2 is the first cytokine secreted by activated T lymphocytes. It binds to IL-2R which is overexpressed after activation and leads to the stimulation of a set of complex signal transduction pathways that produces cell proliferation (Nelson & Willerford, 1998). IL-4 is secreted later by activated T lymphocytes (Th2) and helps B cells to proliferate and differentiate in antibody secreting cells (Constant & Bottomly, 1997).

Materials and methods

Reagents

(−)-Epicatechin, phorbol 12-myristate 13-acetate (PMA), 30 % hydrogen peroxide, propidium iodide, 2-mercapto-ethanol and *o*-phenylenediamine dihydrochloride were obtained from Sigma-Aldrich (Madrid, Spain). Dulbecco's Modified Eagle Medium High Glucose, fetal calf serum (FCS), glutamine and mixture of penicillin–streptomycin–amfoterycin B were purchased from PAA (Pasching, Austria). Arginine was provided by Invitrogen (Prat del Llobregat, Spain). IL-1α, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD25 (IL-2Rα chain, p55) monoclonal antibody (mAb), rat anti-mouse CD16/CD32 (Fcγ III/II receptor) mAb, mouse IL-2 ELISA set and mouse IL-4 ELISA set were obtained from BD Biosciences (Heidelberg, Germany). Sodium azide was provided by Merck (Darmstadt, Germany).

Cocoa extract preparation

Natural Forastero cocoa powder from Malaysia provided by Nutrexpa (Barcelona, Spain) was used. Cocoa was subjected to an extraction of phenols following the Andrés-Lacueva *et al.* (2000) method with some modifications. Briefly, 10 g cocoa were mixed with 8 ml deionised water at boiling point and vortexed for 1 min. Then, 32 ml ethanol were added and the solution was shaken and vortexed for 3 min. The solution was centrifuged for 15 min at 1800 ***g*** at 4°C. The supernatant was concentrated under vacuum to a final volume of 4 ml, avoiding light exposure. To clean the extract, the sample was applied to a Water Oasis HLB extraction cartridge and washed with water and 5 % ethanol in water. The phenols were eluted with ethanol and the extract was evaporated under a stream of nitrogen, avoiding dryness, and reconstituted to 4 ml with ethanol–water (80 %, v/v). The total phenolic content was determined in the extract according to the Folin–Ciocalteu method (Swain & Hillis, 1969). The cocoa extract obtained and used in this study contained 20·4 mg/ml total polyphenols expressed as catechin. This extract was pooled and stored at −80°C until used. Stock solutions of (−)-epicatechin were prepared in a minimum volume of ethanol and then were pooled and stored at −80°C. Just before the treatment of the EL4.BU.OU6 cell line, cocoa polyphenolic extract and (−)-epicatechin ethanolic solutions were appropriately diluted in fresh culture medium in order to obtain the desired concentrations in cell suspensions. Total polyphenol concentrations in the cocoa extract ranged between 5 and 80 μg/ml. Epicatechin was used at final concentrations of 200 and 400 μM, corresponding to 58 and 116 μg/ml of this polyphenol. As cocoa extract and epicatechin were applied to non-physiological conditions in transformed cells, concentrations used were higher than physiological ones.

Treatment of EL4.BU.OU6 cell line with epicatechin and cocoa polyphenolic extract

The thymoma murine EL4.BU.OU6 cell line was provided by ECACC (European Collection of Animal Cell Cultures, Wiltshire, UK). It was maintained in Dulbecco's Modified Eagle Medium High Glucose supplemented with 10 % FCS, 2 mM-glutamine, 0·05 mM-2-mercapto-ethanol, 1·16 g/ml L-arginine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amfoterycin B. Cells were grown in culture flasks at 37°C and 5 % CO_2 atmosphere in humidified air.

To study the influence of cocoa extract on EL4.BU.OU6 cell line, cells were resuspended at 1×10^6 per ml in medium containing 1 or 10 % FCS and incubated in a twelve-well plate. Two hours later, cells were activated by the addition of 10 ng/ml PMA and 0·2 ng/ml IL-1α or remained unstimulated. Immediately, resting and stimulated cells were incubated in the presence of (−)-epicatechin at 200 μM (58 μg/ml) and 400 μM (116 μg/ml) or cocoa extract at 5, 10, 20, 40 and 80 μg/ml. Addition of flavonoids was performed in low light. Controls were incubated with flavonoid vehicle (ethanol). In some experiments, flavonoids were added 2 h before PMA stimulation. After a 24 h activation period, cells were harvested and centrifuged (500 ***g***, 5 min, 4°C). Pellets were immediately used to study cell viability and CD25 expression. Supernatants were stored at −80°C until IL-2 and IL-4 quantification by ELISA.

Cell viability and CD25 expression assay by flow cytometry

Cells were incubated with rat anti-mouse CD16/CD32 mAb (15 min, 4°C) in order to avoid non-specific binding. Cells were

then washed twice with phosphate-buffered saline solution containing 1 % FCS and 0·09 % NaN_3 (PBS-FCS-Az). Cells were labelled with a saturating concentration of FITC-anti-CD25 (30 min, 4°C in darkness). Irrelevant monoclonal antibody of the same isotype as anti-CD25 (IgG1) was used as negative control for each treatment. Cells were washed twice and resuspended with PBS-FCS-Az. Propidium iodide was added to label dead cells. Immediately, cells were analysed on a Coulter Epics XL2 Corporation Fluorescence-Activated Cell Sorter (Miami, FL, USA).

Mean fluorescence intensities (MFI) and percentages of PI-stained cells were assessed by cytometer software (System II Software v.3·0, Coulter Corp.). Viable cells were selected according to the side and forward scatter characteristics of EL4.BU.OU6 cell line and propidium iodide fluorescence. MFI, which are proportional to the amount of CD25 on each cell, were obtained from the analysis of 6000 viable cells. In order to discard the unspecific fluorescence due to resting and activation conditions or flavonoid presence, results from the cytometer were expressed as follows:

$$\text{Percentage of CD25 expression} = F/C \times 100$$

where $C = (\text{MFI}_{\text{CD25}} - \text{MFI}_{\text{IgG1}})_{\text{stimulated cells*}} - (\text{MFI}_{\text{CD25}} - \text{MFI}_{\text{IgG1}})_{\text{resting cells*}}$ and $F = (\text{MFI}_{\text{CD25}} - \text{MFI}_{\text{IgG1}})_{\text{stimulated cells+flavonoids*}} - (\text{MFI}_{\text{CD25}} - \text{MFI}_{\text{IgG1}})_{\text{resting cells+flavonoids*}}$ (*MFI after staining with FITC-conjugated anti-CD25 (or IgG1) mAb obtained from stimulated (or resting) cells.)

IL-2 and IL-4 quantification by ELISA

Levels of IL-2 and IL-4 secreted by EL4.BU.OU6 cells after 24 h of PMA and IL-1α activation were measured using an ELISA sandwich assay. Briefly, an appropriate dilution of anti-mouse IL-2 or anti-mouse IL-4 mAb was incubated on a ninety-six-microwell polystyrene plate overnight at 4°C. Unbound antibodies were washed away using PBS with 0·05 % Tween-20 and the plate was blocked with PBS–10 % FCS (1 h, room temperature). Then, supernatants and standard cytokine dilutions were added (3 h, room temperature). The plate was washed again and a dilution of biotinylated anti-mouse IL-2 or anti-mouse IL-4 mAb and avidine-horseradish peroxidase was added to each well (1 h, room temperature). After washing, cytokines captured by specific mAb were detected by addition of the enzyme substrate (H_2O_2) and a chromogenic solution (*o*-phenylenediamine dihydrochloride). The reaction was stopped after 30 min with 3 M-H_2SO_4. Absorbance was measured at 492 nm. Results were interpolated into standard curves performed with recombinant mouse IL-2 or IL-4.

To study the effect of flavonoids on IL-2 secretion, concentrations of IL-2 secreted by untreated stimulated cells alone was considered as 100 %. The results obtained from stimulated cells in the presence of flavonoids were referred to each parallel untreated stimulated cell.

Statistical analysis

The software package SPSS 10·0 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. A conventional ANOVA was performed with flavonoid concentration as the independent variable and the levels of cytokines and IL-2 receptor (CD25) as dependent variables. When flavonoid content had a significant effect on the dependent variable, a Student's *t* test was performed. Moreover, comparisons between epicatechin and cocoa extract treatments were made by means of Student's *t* test with a significance level of $P < 0{\cdot}05$.

Results

Viability of EL4.BU.OU6 cells after phorbol 12-myristate 13-acetate activation and in flavonoid presence

The effect of cocoa flavonoids on cell viability was determined in resting and stimulated lymphocytes after a 24 h incubation period and under two different media conditions (1 and 10 % FCS supplementation). Epicatechin (200, 400 μM) and cocoa polyphenolic extract (5–80 μg/ml) were tested. Cell viability was reduced from 90 % to 65 % by PMA and IL-1α stimulation in both 1 and 10 % FCS media conditions. Epicatechin did not modify cell viability when it was added in both FCS culture conditions. In 1 % FCS, cell viability was not affected by any of the cocoa extract concentrations tested. In contrast, at 10 % FCS condition, cocoa extract improved viability about 8 %.

IL-2Rα (CD25) expression of EL4.BU.OU6 cells after phorbol 12-myristate 13-acetate activation and in flavonoid presence

CD25 expression was only assessed in viable cells. EL-4.BU.OU6 cells showed low CD25 expression in the resting state (Fig. 1) but it increased two to five times after 24 h activation with PMA and IL-1α in culture conditions of 1 or 10 % FCS (Fig. 1).

Fig. 2 summarises the effect of epicatechin and cocoa extract on IL-2Rα expression of stimulated EL-4.BU.OU6 cells in culture conditions of 10 and 1 % FCS. When cells were stimulated and simultaneously treated with flavonoids in the richest condition (10 % FCS), CD25 expression was not significantly modified with the exception of one concentration (20 μg/ml cocoa extract; $P < 0{\cdot}01$; Fig. 2(A)). On the contrary, under 1 % FCS, CD25 expression was significantly reduced in the presence of 400 μM-epicatechin ($P < 0{\cdot}05$) and 10–80 μg/ml cocoa extract ($P < 0{\cdot}01$; Fig. 2(B)). The highest inhibition achieved was about 40 % both for 400 μM-epicatechin (that corresponds to 116 μg/ml polyphenols) and cocoa extract at concentrations ranging between 10 and 40 μg/ml polyphenols.

To ascertain whether time of flavonoid addition influenced their inhibitory effect, CD25 expression was subsequently studied after addition of epicatechin and cocoa extract 2 h before PMA

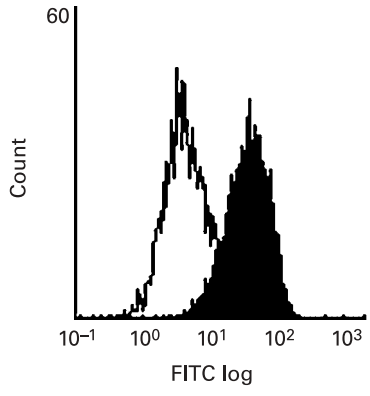

Fig. 1. Representative histogram of resting (□) and 24 h phorbol 12-myristate 13-acetate-activated (■) EL4.BU.OU6 cells after staining with fluorescein isothiocyanate (FITC)-anti-CD25 monoclonal antibody.

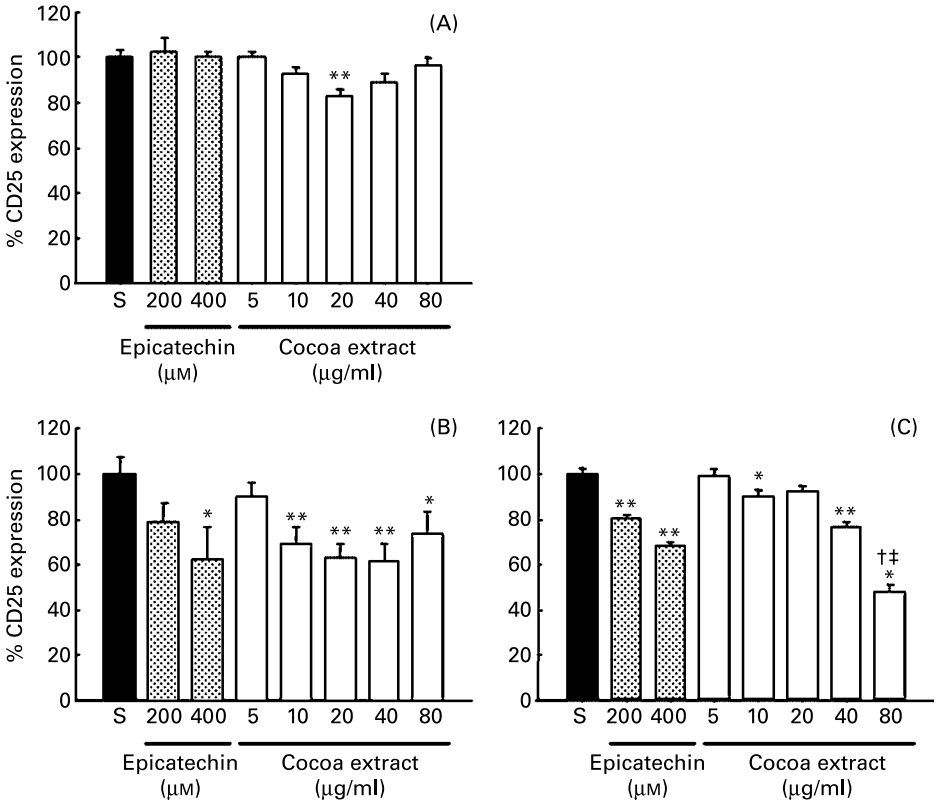

Fig. 2. Effects of epicatechin and cocoa extract on IL-2 receptor α (CD25) expression of activated EL4.BU.OU6 cells when added simultaneously to phorbol 12-myristate 13-acetate (PMA) in conditions of 10 % fetal calf serum (FCS; (A)) and 1 % FCS (B); and (C), when added 2 h previously in conditions of 1 % FCS. Each bar in (A) and (B) represents the mean of ten values with their standard error of the mean obtained from three separate experiments. In (C) each bar represents the mean of six values with their standard error of the mean obtained from three individual experiments. Significant differences between control (PMA + IL-1-stimulated cells, ■) and stimulated cells treated with epicatechin (▨) or cocoa extract (□) are marked by $^{*}P<0{\cdot}05$ and $^{**}P<0{\cdot}01$. Significant differences between stimulated cells treated with 200 and 400 μM-epicatechin and stimulated cells treated with cocoa extract are marked by †$P<0{\cdot}01$ and ‡$P<0{\cdot}01$, respectively.

activation in condition of 1 % FCS. Both cocoa extract (10, 40 and 80 μg/ml) and epicatechin (200, 400 μM) decreased CD25 expression on EL4.BU.OU6 cells in a dose-dependent way ($P<0{\cdot}01$; Fig. 2(C)). Cocoa extract produced a higher effect than that of epicatechin. Thus, 80 μg/ml cocoa extract induced a significantly higher inhibition ($P<0{\cdot}01$) than that produced by 200 and 400 μM-epicatechin (that represent 58 and 116 μg/ml polyphenols, respectively). Moreover, cocoa extract achieved an inhibitory effect of about 50 % that was higher than that obtained with simultaneous addition (Fig. 2(B)).

IL-2 secretion of EL-4.BU.OU6 cells after phorbol 12-myristate 13-acetate activation and in flavonoid presence

ELISA analysis of supernatants after 24 h incubation showed undetectable IL-2 levels in resting cells. In these conditions, neither cocoa extract nor epicatechin addition produced IL-2 secretion. PMA stimulation induced IL-2 secretion, which was higher in 10 % FCS (1180·06 (SEM 28·4) ng/ml) than in 1 % FCS-supplemented media (653·96 (SEM 28·7) ng/ml).

Epicatechin (200, 400 μM) reduced significantly IL-2 secretion between 10 % and 25 % ($P<0{\cdot}05$) and there was no difference among inhibition levels at 10 and 1 % FCS culture conditions (Fig. 3(A) and (B), respectively). Cocoa extract (10–40 μg/ml) also had an inhibitory effect in 1 % FCS media, displaying a reduction of 8–15 % ($P<0{\cdot}01$; Fig. 3(B)). When the study was carried out with 10 % FCS, 80 μg/ml cocoa extract was necessary to obtain a significant reduction in IL-2 production (20 % inhibition; $P<0{\cdot}01$; Fig. 3(A)). However, when epicatechin was added to cells 2 h before activation, it was able to inhibit IL-2 secretion up to 60 % ($P<0{\cdot}01$; Fig. 3(C)). Interestingly, cocoa extract, in this condition, produced an IL-2 release inhibition higher than that produced by epicatechin ($P<0{\cdot}01$) and was able to limit IL-2 production to 25 % (Fig. 3(C)).

IL-4 secretion of EL4.BU.OU6 cells after phorbol 12-myristate 13-acetate activation and in flavonoid presence

Supernatants of EL4.BU.OU6 cells were used to quantify IL-4 secretion. Resting cells secreted undetectable amounts of this cytokine. PMA activation during 24 h induced low levels of IL-4 that was about 4·5 (SEM 2·5) pg/ml in conditions of 1 % FCS culture medium and 5·3 (SEM 2·2) pg/ml in 10 % FCS-supplemented medium. In the richest medium, cocoa extract (5 μg/ml) and epicatechin (200–400 μM) enhanced significantly IL-4 release by about 3- and 4·5-fold, respectively ($P<0{\cdot}05$; Fig. 4). Surprisingly, this effect was not observed when the medium was supplemented with 1 % FCS (data not shown). Similarly, when flavonoids were added 2 h before PMA activation, there was no effect on IL-4 production by EL4.BU.OU6 cells (data not shown).

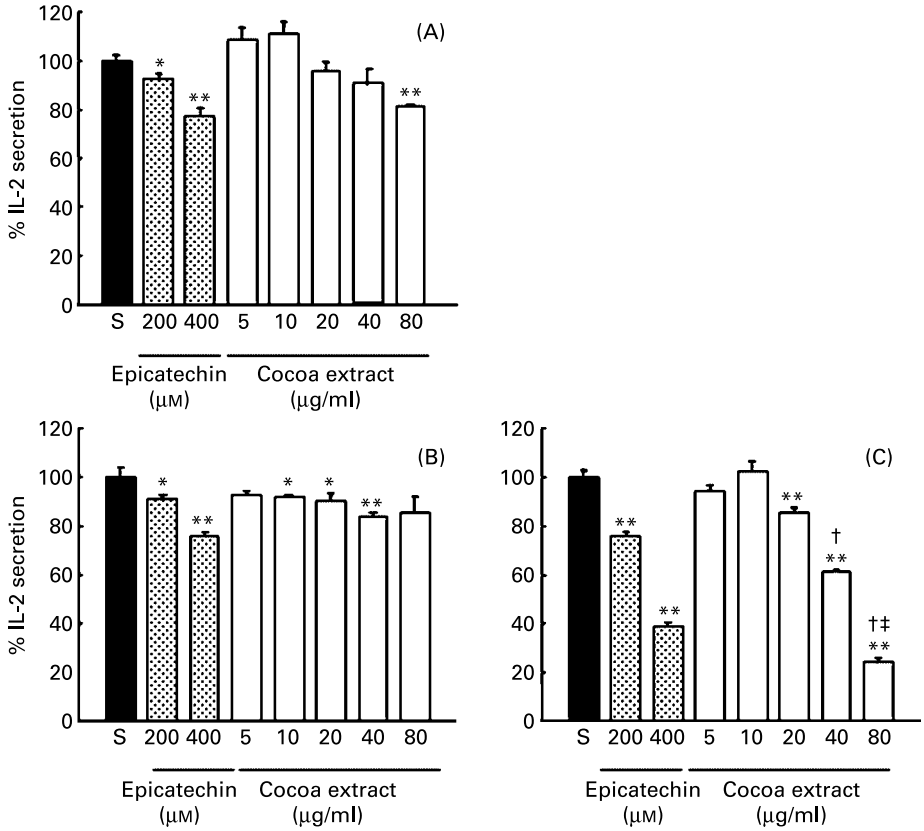

Fig. 3. Effects of epicatechin and cocoa extract on IL-2 secretion of activated EL4.BU.OU6 cells when added simultaneously to phorbol 12-myristate 13-acetate (PMA) in conditions of 10% fetal calf serum (FCS; (A)) and 1% FCS (B); and (C), when added 2 h previously in conditions of 1% FCS. Each bar in (A) and (B) represents the mean of ten values with their standard error of the mean obtained from three separate experiments. In (C) each bar represents the mean of six values with their standard error of the mean obtained from three individual experiments. Significant differences between control (PMA + IL-1-stimulated cells, ■) and stimulated cells treated with epicatechin (▨) or cocoa extract (□) are marked by $^{*}P<0{\cdot}05$ and $^{**}P<0{\cdot}01$. Significant differences between stimulated cells treated with 200 and 400 μM-epicatechin and stimulated cells treated with cocoa extract are marked by †$P<0{\cdot}01$ and ‡$P<0{\cdot}01$, respectively.

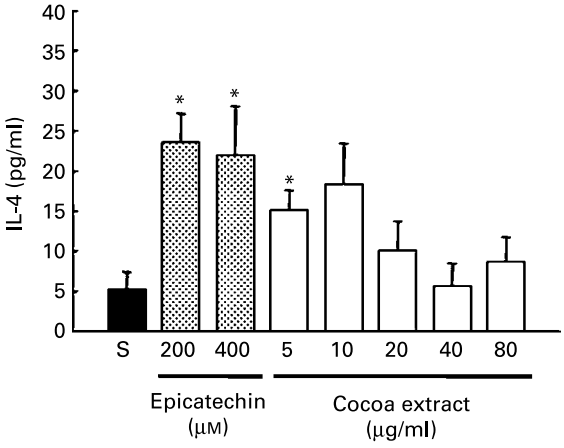

Fig. 4. Effect of epicatechin and cocoa extract on IL-4 secretion of activated EL4.BU.OU6 cells in conditions of 10% fetal calf serum-supplemented media ($^{*}P<0{\cdot}05$). Each bar represents the mean of ten values with their standard error of the mean obtained from three separate experiments. Significant differences between control (phorbol 12-myristate 13-acetate + IL-1-stimulated cells, ■) and stimulated cells treated with epicatechin (▨) or cocoa extract (□) are marked by $^{*}P<0{\cdot}05$.

Discussion

In this study, the down-modulating effects of cocoa flavonoids on T lymphocyte activation have been established. T cell activation occurs in the specific immune response when an antigen is presented by specialised cells, in the context of the main histocompatibility complex class II molecules, to specific, naive T helper (Th) lymphocytes. After the specific recognition of antigenic peptides by T cell receptors, an intracellular signals cascade is triggered that involves the production of IL-2 (Powell *et al.* 1998). IL-2 binds to and signals through a receptor complex consisting of three subunits designated IL-2Rα (CD25), IL-2Rβ (CD122) and IL-2Rγ (CD132) (Nelson & Willerford, 1998). All three subunits are required for high-affinity binding of IL-2. IL-2 by interacting with its specific cell receptor leads to the stimulation of a set of complex signal transduction pathways resulting in cell proliferation (Greene & Leonard, 1986; Hatakeyama & Taniguchi, 1990). In addition to its potent T cell growth-stimulatory activity, IL-2 mediates multiple biological processes, including growth and differentiation of B cells (Waldmann *et al.* 1984). Activated Th lymphocytes can be divided into two distinct subsets of effector cells based on the profile of cytokines that they produce (Constant & Bottomly, 1997). The Th1 subset secretes cytokines usually associated with inflammation, such as IFN-γ and TNF-α and induces cell-mediated immune responses. The Th2 subset

produces cytokines such as IL-4 and IL-5 that help B cells to proliferate and differentiate and is associated with humoral immune responses. Th2 cells also produce IL-10 which suppresses Th1 cells (Constant & Bottomly, 1997). IL-4 is mainly produced by activated Th2 cells and plays an important role in regulating Th1/Th2 balance. It has been found that IL-4 down-regulates Th1 function involved in autoimmune diseases such as psoriasis and arthritis (Allen *et al.* 1993; Ghoreschi *et al.* 2003).

This study was performed in the lymphoid cell line EL4.BOU.OU6 from murine thymoma that can be activated by PMA and IL-1 (Stoeck *et al.* 1989). PMA is a potent protein kinase C activator which causes dose-dependent IL-2 secretion and surface IL-2Rα expression in EL4.BU.OU6 cells. The combined addition of IL-1α with PMA in these cells results in a synergistic increase in IL-4 production. Therefore, PMA addition in EL4.BU.OU6 induces cell activation through a similar pathway to antigen binding to T cell receptors (Dornand *et al.* 1992). This model allowed us to study the effect of an extract from *Theobroma cacao* and the effect of epicatechin alone, in the initial phases of the specific immune activation. Epicatechin was chosen since it is the main flavonoid in cocoa; however, the purified cocoa polyphenolic extract contains a wider flavonoid spectrum (monomers and oligomers; Hammerstone *et al.* 1999) which may explain the differences obtained in some results.

Cocoa extract was able to decrease the surface IL-2Rα (CD25) expression induced by PMA and to diminish IL-2 secretion on the lymphoid line EL4.BU.OU6. We are not aware of any previous studies about cocoa extract and epicatechin on the α chain of the IL-2 receptor (CD25). The decrease of the expression of this receptor could be related to minor production of IL-2, but there are discordant results about this. Thus, some authors have found that IL-2Rα (CD25) expression is induced by IL-2 (Depper *et al.* 1985; Smith & Cantrell, 1985), whereas others fail to observe such a relation (Proust *et al.* 1991; Alileche *et al.* 2001). In any case, treatment of the lymphoid cell line with cocoa extract decreases IL-2Rα (CD25) expression on the lymphocyte surface which results in a cell less sensitive to the autocrine effect of IL-2. The ability of cocoa extract to decrease IL-2Rα (CD25) expression was higher than that of epicatechin alone in some conditions. This fact may be due to the composition of cocoa extract: 40 % epicatechin, catechin and dimer procyanidins, and 60 % trimer-decamer procyanidins (Rios *et al.* 2003). These procyanidins seem to be more active on lymphocytes than epicatechin alone.

Our results about cocoa flavonoids on IL-2 secretion agree with those of Sanbongi *et al.* (1997) who found that cocoa liquor polyphenols inhibited both IL-2 mRNA expression and IL-2 secretion by T cells from human peripheral blood. Moreover, we agree with the results of Mao *et al.* (1999, 2000*c*) that show that crude cocoa extract and pentamer, hexamer and heptamer procyanidin fractions from cocoa extract reduced IL-2 transcription in human peripheral blood lymphocytes. In this sense, our results also show that cocoa extract decreases IL-2 secretion more effectively than epicatechin alone, either demonstrating a greater action of procyanidins or the synergistic effect between cocoa flavonoids.

Other flavonoids produce similar effects on T cell function. Thus, genistein inhibited T cell proliferation, IL-2 synthesis and IL-2Rα (CD25) expression without toxic effects on T cells (Atluru *et al.* 1991). Moreover, low doses of rooibos tea extract have been found to reduce IL-2 secretion from primed murine splenocytes (Kunishiro *et al.* 2001). Furthermore, pycnogenol reduces IL-2 production in stimulated Jurkat E6·1 cells (Cho *et al.* 2001), and kaempferol is able to reduce IFN-γ and IL-2 production by T cells (Okamoto *et al.* 2002). Recently, prenylated flavones from *Artocarpus elasticus* have shown a potent inhibition of IL-2 secretion from stimulated splenocytes (Cerqueira *et al.* 2003) and there exists an *in vivo* study showing that low doses of silymarin have an inhibitory effect on T-lymphocyte function (Johnson *et al.* 2003). Cocoa extract, like other flavonoids, modulates IL-2 secretion in activated lymphocytes. This effect, together with a minor IL-2Rα (CD25) expression, may down-regulate the early activation phase of lymphocyte and, therefore, the subsequent activation of effector lymphoid cells. This could be useful in some states of the exacerbated immune response, as in hypersensitivity and autoimmune diseases.

The effects of epicatechin and cocoa extract on IL-2Rα (CD25) expression and IL-2 secretion were more patent in 1 % FCS than in 10 % FCS medium. In the former condition, cells secreted less IL-2 (about half) than in the latter. This difference in cytokine secretion in 1 % FCS medium may be due to stress from lack of nutrition. Since cells were less responsive to PMA in 1 % FCS medium, it must be easier to modulate them than in 10 % FCS conditions. On the other hand, flavonoids produce a higher inhibitory effect when they are added 2 h before stimulation than when added simultaneously. These 2 h before stimulation may be necessary to enhance the entry of flavonoids into the cell and modulate gene expression. Alternatively, this time period could be necessary to hydrolyse procyanidins from cocoa extract in smaller fractions able to enter into the cell.

The intracellular mechanisms responsible for the effect of cocoa and epicatechin on lymphocyte activation still need to be elucidated. There has been a recent description of the effect of epicatechin and dimeric procyanidins on PMA-activated Jurkat cells, a lymphoid cell line (Mackenzie *et al.* 2004). This study shows that flavonoids, by means of their antioxidant activity or other actions, decrease NF-κB activation. The inhibition of NF-κB might also mediate the down-regulation of IL-2 and IL-2Rα (CD25) expression in our cells. Moreover, a polyphenolic cocoa extract on Caco-2 cell line produces partial overexpression and underexpression of several genes, some of which are involved in the cellular response to oxidative stress whilst others are involved in the expression of cell surface receptors and cell signalling (Noé *et al.* 2004). These data suggest intricate mechanisms of action of cocoa flavonoids at the molecular level.

In the present study, a clear enhancement of IL-4 secretion was found after epicatechin addition and a tendency to increase IL-4 secretion was observed after cocoa treatment. In this sense, Mao *et al.* (1999) also found that monomer cocoa flavonoids enhanced the levels of secretory IL-4 in phytohemagglutinin-stimulated blood mononuclear cells. Moreover, these authors reported that hexamer-octamer procyanidins presented an inhibitory effect on this cytokine and the remaining procyanidins showed no statistically significant effects. These results may explain the higher effect of epicatechin in comparison with cocoa extract observed in the present study. In any case, these results may suggest that cocoa flavonoids could favour Th2 lymphocyte activation. To some degree, when the immune response is askew to Th2 activation, Th1 activity and the inflammatory response are down-regulated reciprocally. The opposite effect of cocoa on IL-2 and IL-4 secretions suggests the action of flavonoids through different pathways involved in cytokine production and secretion by lymphocytes.

In summary, we find that T lymphocyte activation and therefore the specific immune response are down-modulated by cocoa extract and epicatechin. This fact could be important in some states of the immune system hyperactivity such as autoimmune or chronic inflammatory diseases.

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