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Does caffeine reverse the EAC cell-induced immune suppression?

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Abstract

The aim of this study was to investigate the effect of long-term consumption of caffeine in the development of Ehrlich ascites carcinoma (EAC) cells in adult female mice, 25–30 g, in relation to immune response. Mice were treated with caffeine (20 mg kg^{-1} daily, p.o.) for 22–27 consecutive days or inoculated with EAC cells (5×10^6 cells/mL, i.p.), or both. Control mice, corresponding to experimental groups, were treated with corresponding vehicles under similar conditions. The lymphocyte viability, mitogen-induced proliferating activity, cytotoxicity and DNA fragmentation from blood, spleen and thymus of both control and experimental groups were measured as immune response parameters. An immune response index, corticosterone, was also measured in adrenals and plasma under similar conditions. Results showed that development of EAC cells caused immune suppression with a reduction of lymphocyte viability, cytotoxicity and proliferative activity and induction of DNA fragmentation in those tissues, as well as an increase in plasma corticosterone. Though long-term caffeine treatment (which resulted in tolerance to caffeine) alone did not alter significantly any of the immune response parameters studied, including corticosterone status (immune biomarker), the continuation of caffeine treatment during the development of EAC cells either restored or reduced the EAC cell-induced alteration in these parameters, including the HPA axis biomarker. These results suggest that long-term caffeine intake may inhibit or reverse the EAC cell-induced immune suppression.

Introduction

Caffeine, the most potent alkaloid (methylxanthine) of coffee and tea, is widely consumed as a food additive and in beverages all around the world. Structurally, caffeine is similar to several endogenous metabolites, which enables it to be a potentially active pharmacological compound (Kalmar & Cafarelli 1999). Pharmacologically, caffeine primarily mediates its action by antagonizing adenosine receptors (Arnaud 1977) in the central nervous system (CNS) and causes CNS excitability (Gilbert 1984). In recent past reports, authors have shown that caffeine affects the hypothalamo-pituitary-gonadal axis (HPG), hypothalamo-pituitary-adrenal axis (HPA) and central GABA-ergic activity (Spindel et al 1980; Ezzat & El-Gohary 1994; Mukhopadhyay & Poddar 2000; Mandal et al 2007). It has been also noted that caffeine functions as an antioxidant (Mukhopadhyay et al 2003) and antagonizes the development of cancer in various organs, such as lung, breast, etc., as well as Ehrlich ascites carcinoma (EAC) (Theiss & Shimkim 1978; VanderPloeg & Welsch 1991; Mukhopadhyay & Poddar 2001; Conney et al 2002).

It is known that the HPA axis, under controlled signals from the CNS, modulates the immune system through the secretion of adrenocorticotropin (ACTH) from the adrenal hypophysis, which in turn induces the expression and release of glucocorticoids from adrenal glands (George & Chrousos 1995; Turnbull & Rivier 1999). Among the glucocorticoids, corticosterone (in rodents) and cortisol (in man) play a role in the immune response through feedback regulation on the HPA axis and have been considered as the major index of immune response (Besedovsky & Sorkin 1977; Khansari et al 1990; Ashwell et al 1992; Russo-Marie 1992; Black 1994; Elenkov & Chrousos 2002; Saino et al 2003). In addition, the immune system can also modulate the CNS via cytokines released in the periphery (Hopkins & Rothwell 1995; Benveniste 1998; Dallman et al 2004). Further, Straub & Cutolo (2001) and DaSilva (1998) have shown that the HPA axis is affected by oestrogen with the

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changing of the production and secretion of corticotrophin-releasing hormone (CRH). It is also known that the HPG axis plays an important role in the regulation of the immune system in a bi-directional way via the involvement of cytokines, peptide and steroids (Seiki et al 1990; Tranviverdi et al 2005).

Cancer- and other inflammatory-disease-induced stress causes dysfunction of the neuroendocrine regulation of the immune response (Banat et al 2001; Eskandari et al 2003). It is also known that development of implanted inducible tumour is a result of stress (Visintainer et al 1982; Schmitt 2007). It is well known that stress (distress) influences the immune response through the induction of glucocorticoids (Ashwell et al 1992; Russo-Marie 1992; Elenkov & Chrousos 2002). Accordingly, it seems that the change in corticosterone status due to EAC cell-induced stress may have an influence on lymphocyte activity. However, no possible correlation has been made between changes in immune response parameters and glucocorticoid status during the development of EAC cells alone and/or with caffeine treatment. Therefore, the authors are interested to study the effects of long-term exposure to caffeine on immune response in relation to EAC cell development in mice.

Materials and Methods

Chemicals

Caffeine was purchased from Fluca Chemica Biochemica (Switzerland). Histopaque, RPMI-1640, concanavalin A (Con A) and thymidine (cold and ^{3}H-labelled) were purchased from Sigma Chemicals Co. (St Louis, MO). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Anti-galactocerebroside sera was gifted from Prof. C. S. Raine (Albert Einstein College of Medicine, Bronx, USA). All other reagents used were of analytical grade.

Animals

Swiss adult albino female mice, 25–30 g, aged 50–60 days, were used as experimental animals in this study. They were grouped four per cage and housed in a room having 28 ± 0.5°C ambient temperature and 85 ± 5% relative humidity under a 12-h light–dark cycle. All possible care was taken to minimize stress to the mice, with free access to standard diet and water. This study conformed to the guidelines on the ethical use of animals and all possible efforts were followed to minimize the number of mice and their suffering. The animal ethical committee (Registration No. 797/CPCSEA) of the Department of Biochemistry, University of Calcutta approved the use of laboratory animals for experimental purposes.

Experimental design

Mice were grouped and treated accordingly as described elsewhere (Mukhopadhyay & Poddar 2000). They were divided into two groups, each group containing 36 mice. Group 1 was control and group 2 was the experimental group. Each group was further divided into 3 subgroups and each subgroup was further divided into another 3 subgroups (each containing 4 mice). Mice of group 2a were treated with 0.2 mL caffeine (20 mg kg^{-1} daily, p.o.) for 22, 24 and 27 consecutive days. The corresponding control group (1a) was supplemented with an equivalent amount of vehicle (water) through the same route under similar conditions. EAC cells were inoculated to group 2b by a single intraperitoneal administration of 0.2 mL ascites fluid at a concentration of 1×10^7 EAC cells/mL and were allowed to develop for 10, 12 or 15 days. Control group 1b was prepared using normal saline instead of ascites fluid. Group 2c was pretreated with caffeine (20 mg kg^{-1} daily, p.o.) for 12 consecutive days, followed by EAC cell inoculation and caffeine treatment was continued for another 10, 12 or 15 consecutive days. Control group 1c, corresponding to the above experimental group 2c, was prepared using water instead of caffeine and saline instead of ascites fluid using the same route under similar conditions.

Collection of blood and tissues

Mice of both control and experimental groups were sacrificed by cervical dislocation between 0800 and 1000 h to minimize or avoid any circadian effect. During the sacrifice, care was taken to inflict minimum physical pain to the mouse. Mice of the caffeine-treated group were sacrificed 30 min after the last caffeine administration. Immediately after sacrifice, blood was collected in sterile centrifuge tube along with anticoagulant (heparin) and stored at room temperature. Adrenal, thymus and spleen were removed aseptically and collected into small-capped tubes in cold phosphate-buffered saline (PBS) containing 0.01 M phosphate and 0.15 M NaCl, at pH 7.1.

Lymphocyte culture from blood, spleen and thymus

Blood lymphocytes were separated according to the method of Boyum (1968). Uncoagulated blood was mixed with an equal volume of PBS, layered over lymphocyte separating media (1 mL Histopaque) and centrifuged at 800–1200 rev min^{-1} for 45 min. The upper layer (containing lymphocytes) was aspirated, collected and erythrocytes were removed by washing with lysing buffer (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM bi-sodium EDTA) (Shortman et al 1972). Spleen and thymus lymphocyte suspension was prepared following the method described by Perandones et al (1993). Briefly, the tissues were gently teased and passed through a sterile 40-μm nylon mesh screen. The lymphocyte suspensions were then freed from contaminating erythrocytes by NH_4Cl treatment (Shortman et al 1972) followed by elimination of dead cells (Von Boehmer & Shortman 1973) as described for the blood samples. The lymphocytes obtained from blood, spleen and thymus were washed three times with complete medium (RPMI-1640 supplemented with 10% FBS, 2 mM glutamine and 1% penicillin–streptomycin mixture) and re-suspended in the same medium at a concentration of 1×10^6 cells/mL. All the suspensions were incubated at 22°C to remove B cells (Severson et al 1987) and non-adherent cells were cultured at 37°C in 5% CO_2 atmosphere (Nu, Air, TS, Autoflow water-jacketed CO_2 incubator) for 18 h to remove macrophages. After removing adherent macrophages from the culture, the cells suspensions were used for the cytotoxicity and mitogen-induced proliferating activity assays.

Lymphocyte viability test

The lymphocyte viability of blood, spleen and thymus was counted by haemocytometer following the trypan blue exclusion method (0.2% trypan blue in final solution) as described by Modha et al (1993).

Cell-mediated cytotoxicity assay

Lymphocytes were tested for cytotoxicity against anti-galactocerebrosidase (Anti-GC) following the method of Selmaj et al (1991). In brief, the cells (0.1 mL) were incubated with rabbit complement (0.05 mL of 1:10 diluted with complete medium) with or without Anti-GC sera (0.1 mL, 1:100 diluted with complete medium) in a 96-well microtitre plate (Laxbro, India) for 24 h at 37°C in a 5% CO_2 incubator. Thereafter, pellets of antigen–antibody complexes were obtained by centrifugation at 10 000 rev min^{-1} for 10 min and were stained with 0.05 mL 0.2% crystal violet at room temperature. The excess dye was removed by washing with PBS (three times) and finally dissolved by 0.2 mL sodium dodecyl sulfate detergent in 1 mL PBS. The absorbance was measured spectrophotometrically at 540 nm (Hitachi U-2000). Cytotoxicity (%) was expressed as:

$$\text{Cytotoxicity (\%)} = [(1 - \text{OD}_{540} \text{ of control})/\text{OD}_{540} \text{ of experimental}] \times 100 \quad (1)$$

Mitogen (concanavalin A)-induced proliferating activity

Lymphocytes from blood, spleen and thymus were subjected to Con A-induced proliferating assay as described by MacCain et al (1987). The lymphocyte suspensions were incubated with or without Con A (in 96-well microtitre plate, Laxbro India) for 24 h at 37°C in a 5% CO_2 incubator. This lymphocyte culture was further incubated with 3 μCi ^{3}H-thymidine for 24 h at 37°C in a 5% CO_2 incubator. Excess and unbound thymidine was removed by washing with cold thymidine and incorporation of ^{3}H-thymidine in the newly synthesized DNA was observed in a liquid scintillation counter (Wallac) by using cocktail'O as medium. The proliferating activity was expressed in counts per minute (CPM) as:

$$\text{CPM} = \text{Count}_{\text{Experimental}} - \text{Count}_{\text{Control}} \quad (2)$$

DNA fragmentation assay

DNA fragmentation assay was performed according to the method of Perandones et al (1993). Briefly, fresh uncultured cells of lymphocytes from blood, spleen and thymus were prepared as described above. Each sample was resuspended in 400 μL lysis buffer (0.2% Triton X-100, 10 mM Tris and 1 mM EDTA, pH 8.0) and centrifuged at 11 500 rev min^{-1} for 15 min (Newell et al 1996). The small DNA fragments in the supernatant and the large DNA fragments in the pellets were collected (re-suspended in 200 μL lysis buffer) and both treated with 100 μL 0.5 M perchloric acid. Thereafter, 400 μL diphenylamine (DPA) solution (containing 0.088 M DPA, 98% v/v glacial acetic acid, 1.5% v/v sulfuric acid and 0.5% v/v of 1.6% acetaldehyde) was added to each sample and incubated at 4°C for 48 h. Finally, spectrophotometric quantitation was carried out at 575 nm. The DNA fragmentation was expressed as:

$$\text{DNA fragmentation (\%)} = [\text{OD}_{575} \text{ of SN}/(\text{OD}_{575} \text{ of SN} + \text{OD}_{575} \text{ of LN})] \times 100 \quad (3)$$

where SN and LN are small and large nucleotide fragments, respectively.

Estimation of plasma and adrenal corticosterone

Corticosterone in plasma and the adrenals was estimated according to the method of Purves & Sirett (1965). Plasma was obtained from heparinized blood by centrifugation at 2500 rev min^{-1} for 10 min in a UV model centrifuge. Plasma (0.3 mL) was mixed with 2.7 mL distilled water and 7.5 mL chloroform. The mixture was shaken for 15 s and centrifuged at 2500 rev min^{-1} for 5 min. The organic layer was separated. A 4.5-mL volume was mixed with 1 mL 0.1 M NaOH and shaken for 15 s. The aqueous layer was removed and 3 mL of the organic part was mixed with 2 mL fluorescent reagent (2.4 volumes of H_2SO_4 and 1 volume 50% (v/v) aqueous ethanol). After shaking for 15 s, the mixture was centrifuged at 2500 rev min^{-1} for 5 min and the organic layer was completely removed. The mixture was kept at 37°C for 30 min and the fluorescence was measured with excitation at 470 nm and emission at 520 nm using a spectrophotofluorimeter (Hitachi F-3010 model). The corresponding blank and standard were prepared by using water and pure corticosterone instead of plasma. Adrenal gland was homogenized in 20% (v/v) saline–ethanol mixture at 0–4°C. The homogenate was centrifuged at 4000 rev min^{-1} for 19 min in a Sorrall RC-5B model cold centrifuge at 4°C. After discarding the pellet, 1 mL of supernatant fluid was used for extraction of corticosterone as described for plasma. The corticosterone was assayed by following the same procedure as described above. The plasma and adrenal corticosterone level was expressed as μg mL^{-1} and μg (mg protein)$^{-1}$), respectively.

Estimation of protein

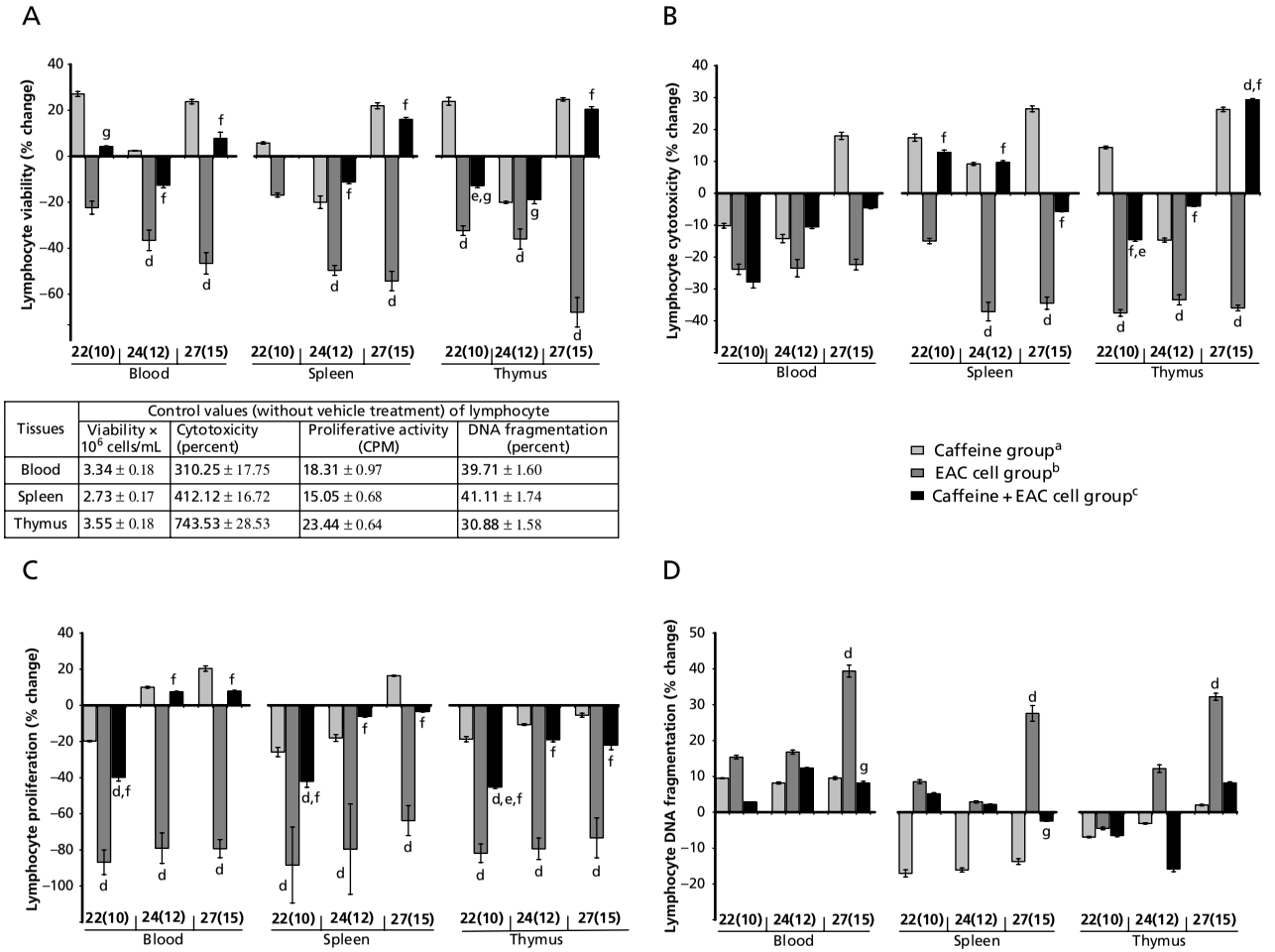
Protein from adrenal gland of both control and experimental mice was estimated following the method of Lowry et al (1951) using bovine serum albumin as standard.

Statistical analysis

Statistical significance between the groups of the experimental results was assessed by two-way analysis of variance.

Results

No significant change in any of the parameters studied, with respect to their corresponding control (without any vehicle treatment), as well as between the groups and duration of treatment with vehicle of caffeine and/or EAC cell, was observed (results not shown). Figure 1 shows that long-term caffeine administration (20 mg kg^{-1} daily, p.o.) for 22, 24 and



Tissues	Control values (without vehicle treatment) of lymphocyte			
	Viability × 10^6 cells/mL	Cytotoxicity (percent)	Proliferative activity (CPM)	DNA fragmentation (percent)
Blood	3.34 ± 0.18	310.25 ± 17.75	18.31 ± 0.97	39.71 ± 1.60
Spleen	2.73 ± 0.17	412.12 ± 16.72	15.05 ± 0.68	41.11 ± 1.74
Thymus	3.55 ± 0.18	743.53 ± 28.53	23.44 ± 0.64	30.88 ± 1.58

Figure 1 Effect of long-term consumption of caffeine on immune parameters (lymphocyte viability (A), cytotoxicity (B), mitogen-induced proliferating activity (C) and DNA fragmentation (D) in blood or spleen or thymus) of mice inoculated with Erlich ascites cells (EAC). Results are expressed as mean ± s.e.m. of four separate observations. Percent changes were calculated with respect to their corresponding control. [a]Caffeine was administered (p.o.) at a dose of 20 mg kg^{-1} daily. The corresponding control group was treated with an equivalent amount of vehicle (water). [b]EAC cells were inoculated (i.p.) from donor mice and allowed to develop for 10, 12 and 15 days. The corresponding control group was injected with sterile saline under similar conditions. [c]The mice were first pretreated with caffeine for 12 consecutive days followed by inoculation with EAC cells (i.p.) and the treatment with caffeine was continued for another 10, 12 and 15 consecutive days. Control group corresponding to the experimental group was treated with vehicle (water) instead of caffeine and sterile saline instead of EAC through the same route under similar condition(s). The number in parentheses indicates the duration of EAC cell development. bars represents ± s.e.m. Significantly different from the corresponding control group: [d]$P < 0.01$, caffeine group [e]$P < 0.01$, cancer group [f]$P < 0.01$, [g]$P < 0.05$ (using analysis of variance).

27 consecutive days caused no significant alteration in lymphocyte viability, cytotoxicity, mitogen-induced proliferating activity or DNA fragmentation in blood or spleen or thymus with respect to their corresponding control. Figure 1 also shows that the EAC cell development in mice for 10, 12 and 15 days significantly ($P<0.01$) decreased lymphocyte viability, mitogen-induced lymphocyte proliferation and its cell-mediated cytotoxicity in blood, spleen and thymus, except viability in blood and spleen for 10 days development and cytotoxicity in blood during 10 to 15 days development, with respect to their corresponding controls. The present reduction of these parameters in a particular tissue is not significantly altered during 10–15 days development of EAC cells. Unlike those parameters mentioned above, the DNA fragmentation (Figure 1D) was significantly ($P<0.01$) increased in blood, spleen and thymus for only 15 days (but not for 10 or 12 days) development of EAC cells with respect to the corresponding control.

It is evident from Figure 1A, B and D that pretreatment with caffeine (20 mg kg^{-1} daily; p.o.) for 12 consecutive days followed by EAC cell inoculation and continuation of caffeine treatment at the same daily for another 10, 12 and 15 consecutive days during the EAC cell development (1) caused no appreciable alteration in lymphocyte viability, cytotoxicity and DNA fragmentation of blood, spleen or thymus during (10–15 consecutive days) caffeine treatment, but significantly reduced mitogen-induced lymphocyte proliferating activity (Figure 1C) in blood ($P<0.01$), spleen ($P<0.01$) and thymus ($P<0.01$) with respect to their corresponding controls. Lymphocyte viability was also significantly decreased (30.05%; $P<0.01$) along with its cytotoxicity (26.08%, $P<0.01$) and proliferating activity in thymus (31.89%, $P<0.01$) without affecting blood and spleen following EAC cell development for 10 days with respect to their corresponding caffeine treated group (Figure 1A–C). The rest of the parameters in the remaining tissues during other durations of caffeine treatment were not significantly changed under similar condition (Figures 1A–D) and (2) significantly (Figure 1A) (a) increased: firstly, lymphocyte viability in blood (28.84%, $P<0.05$) and thymus (29.24%, $P<0.05$) following 10 days, in blood (38.14%, $P<0.01$ and 101.12%, $P<0.01$, respectively), spleen (76.98%, $P<0.01$ and 141.09%, $P<0.01$, respectively) and thymus (28.14%, $P<0.05$ and 261.21%, $P<0.01$, respectively) following 12 and 15 days of EAC cell development; secondly, cytotoxicity in spleen and thymus, but not blood, following 10 (33.66%, $P<0.01$ and 35.45%, $P<0.01$ respectively), 12 (73.52%, $P<0.01$ and 45.22%, $P<0.01$, respectively) and 15 (44.93%, $P<0.01$ and 100.11%, $P<0.01$, respectively) days; and thirdly, lymphocyte proliferation in blood, spleen and thymus following 10 (353.31%, $P<0.01$, 418.45%, $P<0.01$ and 206.47%, $P<0.01$, respectively), 12 (411.70%, $P<0.01$, 297.76%, $P<0.01$ and 373.18%, $P<0.01$, respectively) and 15 (419.58%, $P<0.01$, 165.36%, $P<0.01$ and 195.99%, $P<0.01$, respectively) days of EAC cell development, and (b) decreased lymphocyte DNA fragmentation in blood (22.78%, $P<0.05$) and spleen (28.02%, $P<0.05$), but not thymus, following only 15 days of EAC cell development with respect to corresponding EAC cell developing condition alone.

Table 1 shows that caffeine treatment alone (20 mg kg^{-1} daily, p.o.) for 22–27 consecutive days did not change the corticosterone levels in both plasma and adrenal gland with respect to their corresponding control. However, the level of corticosterone was significantly decreased during the development of EAC cells for 10 (63.54%, $P<0.01$), 12 (58.25%, $P<0.01$) and 15 (48.73%, $P<0.01$) days in adrenal gland but

Table 1 Effect of long-term caffeine consumption on EAC cell-induced change in corticosterone levels in plasma and adrenals of mice

Experimental condition(s)	Period of caffeine treatment and/or development of EAC cells (days)	Corticosterone level			
		Plasma (μg mL^{-1})		Adrenal (μg (mg protein)$^{-1}$)	
		Control	Experimental	Control	Experimental
Caffeine[a]	22	0.135 ± 0.010	0.146 ± 0.011	0.194 ± 0.011	0.181 ± 0.010
	24	0.131 ± 0.027	0.125 ± 0.027	0.190 ± 0.012	0.203 ± 0.011
	27	0.137 ± 0.010	0.151 ± 0.020	0.194 ± 0.014	0.210 ± 0.016
EAC cell development[b]	10	0.130 ± 0.014	0.215 ± 0.010[d]	0.201 ± 0.010	0.066 ± 0.007[d]
	12	0.146 ± 0.014	0.263 ± 0.019[d]	0.194 ± 0.018	0.081 ± 0.008[d]
	15	0.135 ± 0.008	0.248 ± 0.029[d]	0.197 ± 0.022	0.101 ± 0.012[d]
Treatment with caffeine during EAC cell development[c]	10 (22)[1]	0.132 ± 0.015	0.142 ± 0.013[e]	0.198 ± 0.012	0.148 ± 0.014[e]
	12 (24)[1]	0.138 ± 0.010	0.150 ± 0.016[e]	0.195 ± 0.016	0.149 ± 0.014[e]
	15 (27)[1]	0.131 ± 0.014	0.145 ± 0.027[e]	0.197 ± 0.027	0.182 ± 0.012[e]

Results were expressed as mean ± s.e.m. of 4 separate observations. [a]Caffeine treatment (20 mg kg^{-1} day, p.o.) was given for 22, 24 and 27 consecutive days. The corresponding control groups were treated with equivalent amount of vehicle (water) of caffeine for the same duration. [b]EAC cells were inoculated (i.p.) from donor mice and allowed to develop for 10, 12 and 15 days. The corresponding control groups were injected with an equivalent amount of sterile saline under the same conditions. [c]The mice were first pretreated with caffeine for 12 consecutive days followed by the inoculation of EAC cells (i.p.) and the treatment with caffeine was continued for another 10, 12 and 15 consecutive days. Control group corresponding to the experimental group was treated with vehicle of caffeine (water) instead of caffeine and sterile saline instead of EAC through the same route and under similar condition(s). [1]The number in the parentheses indicates the duration of EAC cell development. [d]$P<0.01$ compared with corresponding control; [e]$P<0.01$ compared with EAC cell development group.

increased in plasma (65.38%, $P<0.01$; 80.14%, $P<0.01$; 83.70%, $P<0.01$, respectively) with respect to their corresponding controls. Further, it was noted that these EAC-induced changes of corticosterone in adrenal and plasma were enhanced with duration of development. Caffeine treatment to mice for 12 consecutive days before EAC cell inoculation followed by continuation of its treatment for another 10, 12 and 15 consecutive days during the development of EAC cells caused no significant alteration in either plasma or adrenal corticosterone levels with respect to their corresponding control and also with respect to the corresponding caffeine-alone treatment group. However, this treatment caused a decreased plasma corticosterone level (33.95%, $P<0.01$; 42.97%, $P<0.01$; 41.53%, $P<0.01$, respectively) and increased adrenal corticosterone level (122.24%, $P<0.01$; 83.95%, $P<0.01$; 80.20%, $P<0.01$, respectively) with the increase of 10–15 days of development with respect to the corresponding group of only EAC cell bearing mice. Further, it is observed that the effect of caffeine on EAC cell-induced decrease of plasma corticosterone and increase of adrenal corticosterone were potentiated with increase in duration (22–27 consecutive days) of caffeine treatment.

Discussion

The immune system is known to be affected by a variety of physical, chemical and psychological stressors (Otaviani & Franceschi 1996). Caffeine is a well-known psychoactive chemical stimulant (Fisone et al 2004). The restraining effect of caffeine on a variety of cancers such as ovarian, lung, skin and breast cancer, including Ehrlich ascites tumour development, has been reported from our laboratory (Mukhopadhyay & Poddar 2000, 2001; Mandal et al 2007) as well as by others (Theiss & Shimkim 1978; VanderPloeg & Welsch 1991; Conney et al 2002).

Mukhopadhyay & Poddar (2000) and Mandal et al (2007) have also reported that caffeine may mediate its action through interaction with the HPA- and HPG-axis involving excitatory and inhibitory neurotransmitters, neuropeptides and neurotrophic hormones, including LH and FSH. Ramanaviciene et al (2004) have shown that caffeine protects non-specific immune response. Further, it is also known that cancer causes stress (Banat et al 2001). It affects the immune system both at the cellular and humoral levels (Hadden 2003). Thus, it is not unreasonable to assume that cancer may exert its action as a stress inductor differently at different tissues of the immune system existing in different immune compartments and hence may affect the compartmentalized immune response profile. Therefore, the authors were interested in studying the action of caffeine on thymus and spleen (as the two major immune organs) and blood in relation to EAC cell development at the level of lymphocyte viability, cytotoxicity, mitogen-induced proliferative activity, and its DNA fragmentation as immune response parameters and corticosterone status as a biomarker of immune response (Besedovsky & Sorkin 1977; Khansari et al 1990).

The EAC cell-induced suppression of immune response due to decrease in T lymphocyte viability, mitogen-induced proliferating activity, cytotoxicity and increase in its DNA fragmentation in both the immune organs (spleen and thymus) and blood (except cytotoxicity) (Figure 1) may be considered as an effect of increase in plasma corticosterone level (Table 1) due to tumour-induced initiation and prolongation of cellular stress (Hadden 2003; Ronson 2006). The increase in plasma corticosterone may be explained by the stimulation of hypothalamic ACTH-induced greater production and release of adrenal corticosterone (George & Chrousos 1995; Turnbull & Rivier 1999). It is known that stress suppresses the immune function via the interaction between the nervous system, endocrine system and immune system in a bi-directional pathway through regulating the production and release of glucocorticoids as well as catecholamines, endogenous opioids, growth hormone and cytokines (Dantzer & Kelly 1989; Khansari et al 1990). Ehrlich ascites tumour-induced stress may be a possible reason for stimulation of hypothalamic ACTH release. However, the results shown in Figure 1 suggest that the degree of immune suppressive effect due to EAC cell development depends on the type of tissue and the period of tumour development. The greater reduction of spleen lymphocyte viability than that of blood and thymus suggests the possible primary invasion of EAC cells through the lymphatic channel as observed in other cancer cells (Visintainer et al 1982; Fidlar 1997), which in turn may result in the death of more lymphocytes in spleen than in blood and thymus. The early reduction of thymocyte viability before splenocytes and blood lymphocytes during tumour progression (Figure 1A) seems to be due to (1) there being fewer active T cells in the thymus than in the blood and spleen (Beretta 2004), and/or (2) to depletion of the thymic pool of lymphocytes as a result of imbalance between B and T lymphocyte in the peripheral system (Beretta 2004) and/or to redistribution of lymphocyte subset population in the different compartments of the immune system under stressed condition (Lourdes & Manuel 2001). Similarly early reduction of thymocyte cytotoxicity in comparison with splenocytes and lymphocytes may be explained as described above. However, no significant change was observed in blood lymphocyte cytotoxicity under the present experimental condition (Figure 1B), possibly due to the alteration of T cell subsets in different compartments of the body under stressful condition (Lourdes & Manuel 2001). The reduction in mitogen-induced lymphocyte proliferating activity in blood, spleen and thymus (Figure 1C) during EAC cell development may manifest the marked reduction in immune response due to the progression of tumour inside the system. The EAC cell-induced significant increase in DNA fragmentation following only 15 days development (not at the earlier stage) of EAC cells (Figure 1D) in blood, spleen and thymus may indicate the inclination of the system to collapse (Mandal el al 2005). This may be explained by decrease in cell viability, increase in cell death of lymphocytes (more significantly the T lymphocytes) or atrophy of immune glands during tumour development (Carr 1983; Thome & Tschopp 2001).

Though caffeine treatment for short periods increases both plasma and adrenal corticosterone levels (Spindal et al 1983; Mukhopadhyay & Poddar 1998), long-term consumption for more than 12 consecutive days restored the short-term caffeine-induced increase in corticosterone status to the normal level (Mukhopadhyay & Poddar 1998). This may be due to

the development of tolerance to caffeine, which has been supported by the observations at the level of locomotor activity as well as by hypothalamic GABA-ergic activity under similar conditions of caffeine treatment with respect to the corresponding control (Mukhopadhyay & Poddar 1998, 2000, 2001). The insignificant correlation between caffeine consumption (for 22–27 consecutive days) and immune response parameters studied under corresponding condition (Table 1, Figure 1) suggests that under the present experimental condition, the immune system, like the endocrine system and nervous system (Banat et al 2001; Eskandari et al 2003), may develop tolerance to caffeine. This is in accordance with the results reported by Carney (1982) and Holtzman & Finn (1988), in which caffeine treatment for more than 12 consecutive days showed tolerance to the HPA axis.

The long-term caffeine treatment during the development of EAC cell, on the other hand, restored the EAC cell-induced dysfunction of all the immune response parameters (Figure 1), including immune index (Table 1), under the present experimental condition (except mitogen-induced lymphocyte proliferating activity following 22 consecutive days of caffeine treatment). However, the reappearance of EAC cell-induced dysfunction of immune response parameters following EAC cell inoculation to mice already tolerant to caffeine (following 12 consecutive days of caffeine treatment) (Figure 1), except the proliferative activity for 22 consecutive days of caffeine treatment, signifies the potential of caffeine to redirect the mice from being tolerant to reverse-tolerant (Casas et al 1999). The reverse-tolerance, in turn, may suppress tumour-induced reduction of immune response via the down-regulation of EAC cell-induced stimulation of the HPA- or sympatho-adrenal-medullary (SAM)-axis, which finally leads to immune potentiation. The down-regulation of the HPA-axis may be achieved via the supersensitization of EAC cell-mediated stress-induced desensitized glucocorticoid receptor (Oakley & Cidlowski 1993; Deak et al 1999). Caffeine-induced down-regulation of the HPA-axis during tumour-induced stress may reduce the production of CRH from the hypothalamus, which in turn reduces the EAC cell-mediated stress-induced stimulation of ACTH release towards normal. Subsequently, the controlled release of ACTH in the blood stream directs the production of corticosterone towards normal in the adrenal gland in a time-dependent manner (Table 1), which in turn may regulate a wide variety of immune functions, including the modulation of cytokine expression, cell proliferation, cellular-trafficking, chemo-attractant expression, adhesion-molecule expression, etc. (Ashwell et al 1992; Russo-Marie 1992; Besedovsky & Sorkin 1977; Khansari et al 1990; Black 1994; Saino et al 2003; Elenkov & Chrousos 2002). In a parallel way, caffeine, under its reverse-tolerant condition, may also down-regulate cancer cell-induced chronic activation of the SAM-axis, which leads to normalization of the immune response through the synaptic innervations of nerve terminals into the lymphoid organs (Felten et al 1992) and may modulate a variety of immune functions, including cellular proliferation, trafficking, antibody and cytokines production and cytolytic activity, etc. (Sanders & Kohm 2002; Madden 2003). It is also evident that the effect of caffeine in normalizing EAC cell-induced dysregulated (Table 1, Figure 1) immune function in tumour-bearing mice becomes prominant with time, indicating its beneficial effect under long-term exposure. Moreover, functional similarity between cortisol and corticosterone in relation to both the HPA- and SAM-axis (Harbuz 2002; Raubenheimer et al 2006) may signify the implication of the present knowledge to humans. Since rodents are less sensitive and more tolerant to drug(s) than humans (Gupta et al 1986; Hengstler et al 2004), further clarification is needed at the level of tolerance and sensitivity to drug(s), with respect to their dosage, before application in human health.

Further work in relation to, firstly, the involvement of other immune-regulatory molecules (like cytokines) and their receptors, neuropeptides and substance P, secondly, lymphocyte subset profile along with the other immune response parameters and, thirdly, the interaction of the brain system at the neuroendocrine interface may allow exploration of the possible mechanisms in more detail.

Conclusion

In conclusion, it may be stated that caffeine exposure under its tolerant condition did not affect the immune response in mice but continuation of its exposure during tumour (induced after the onset of caffeine tolerance) development may suppress or restore the EAC cell-mediated stress-induced dysfunction of immune response under its reverse-tolerant condition via down regulation of the HPA-axis in relation to the involvement of corticosterone in mice.

References

Arnaud, M. J. (1977) The pharmacology of caffeine. *Prog. Drug. Res*. **31**: 273–313

Ashwell, J. D., Lu, F. W., Vacchio, M. S. (1992) Glucocorticoid in T cell function. *Annu. Rev. Immunol*. **18**: 309–345

Banat, G. A., Christ, O., Cochlovius, B., Pralle, H. B., Zoller, M. (2001) Tumor-induced suppression of immune response and its correction. *Cancer-Immunol. Immunother*. **49**: 573–586

Benveniste, E. N. (1998) Cytokines action in the central nervous system. *Cytokines Growth Factor Rev*. **9**: 259–275

Beretta, L. (2004) Translational control in T-lymphocytes. *Int. Rev. Immunol*. **23**: 347–363

Besedovsky, H., Sorkin, E. (1977) Network of immune-neuroendocrine interactions. *Clin. Exp. Immunol*. **27**: 1–12

Black, P. H. (1994) Central nervous system-immune system interactions: psychoneuroendocrinology of stress and its immune consequences. *Antimicrob. Agents Chemother*. **38**: 1–6

Boyum, A. (1968) Isolation of leukocytes from human blood. Further observations. *Scand. J. Clin. Invest*. **21**: 31–50

Carney, J. M. (1982) Effects of caffeine, theophylline and theobromine on scheduled controlled responding in rats. *Br. J. Pharmacol*. **75**: 451–454

Carr, I. (1983) Lymphatic metastasis. *Cancer Metastasis Rev*. **2**: 307–317

Casas, M., Prat, G., Robledo, P., Barbanoj, M., Kulisevsky, J., Jane, F. (1999) Repeated co-administration of caffeine and bromocriptine prevents tolerance to the effects of caffeine in the turning behavior animal model. *Eur. Neuropsychopharmacol*. **9**: 515–521

Conney, A. H., Lu, Y. P., Lou, Y. R., Huang, M. T. (2002) Inhibitory effects of tea and caffeine on UV-induced carcinogenesis:

relationship to enhance apoptosis and decreased tissue fat. *Eur. J. Cancer Prev*. **11**: S28–S36

Dallman, M. F., Akana, S. F., Strack, A. M., Scribner, K. S., Pecoraro, N., La Fleur, S. E., Houshyar, H., Gomez, F. (2004) Chronic stress-induced effects of corticosterone on brain: direct and indirect. *Ann. N.Y. Acad. Sci*. **1018**: 141–150

Dantzer, R., Kelley, K. W. (1989) Stress and immunity: an integrated view of relationships between the brain and the immune system. *Life Sci*. **44**:1995–2008

DaSilva, J. A. (1998) Sex hormone and glucocorticoids: interaction with the immune system. *Ann. NY Acad. Sci*. **876**: 102–117

Deak, T., Nguyen, K. T., Cotter, C. S., Fleshner, M., Watkins, L. R., Maier, S. E., Spencer, R. L. (1999) Long-term changes in mineralocorticoid and glucocorticoid receptor occupancy following exposure to an acute stressor. *Brain Res*. **847**: 211–220

Elenkov, I. J., Chrousos, G. P. (2002) Stress hormone, proinflammatory and antiinflammatory cytokines, and autoimmunity. *Ann. N.Y. Acad. Sci*. **966**: 290–303

Eskandari, F., Webster, J. I., Sternberg, M. (2003) Neural immune pathway and their connection to inflammatory disease. *Arthritis Res. Ther*. **5**: 251–265

Ezzat, A. R., El-Gohary, Z. M. (1994) Hormonal and histological effects of chronic caffeine administration on the pituitary-gonadal and pituitary-adrenocortical axis in male rabbits. *Funct. Dev. Morphol*. **4**: 45–50

Felten, S. Y., Felten, D. L., Bellinger, D. L., Olschowka, J. A. (1992) Noradrenergic and peptidergic innervation of lymphoid organ. *Chem. Immunol*. **52**: 25–48

Fidlar, I. J. (1997) Molecular biology of cancer: invasion and metastasis. In: DeVita, V. T., Hellman, S., Rosenberg, S. A. (eds) *Cancer: principles and practice of oncology*. Lippincott-Raven, Philadelphia, PA, pp 135–152

Fisone, G., Borqkvist, A., Usiello, A. (2004) Caffeine as a psychomotor stimulant: mechanism of action. *Cell. Mol. Life Sci*. **61**: 857–872

George, P., Chrousos, M. D. (1995) The hypothalamic–pituitary–adrenal axis and immune-mediated inflammation. *N. Engl. J. Med*. **332**: 1351–1363

Gilbert, R. M. (1984) Caffeine consumption. In: Spiller, G. A. (ed.) *The methylxanthine beverages and foods: chemistry, consumption, and health effects*. Liss, New York, pp 108–124

Gupta, R. S., Chopra, A., Stetsko, D. K. (1986) Cellular basis for the species differences in sensitivity to cardiac glycosides (digitalis). *J. Cell. Physiol*. **127**: 97–206

Hadden, J. W. (2003) Immunodeficiency and cancer. *Int. Immunopharmacol*. **3**: 1061–1071

Harbuz, M. (2002) Neuroendocrine function and chronic inflammatory stress. *Exp. Physiol*. **87**: 519–525

Hengstler, J. G., Burg, B. V. D., Steinberg, P., Oesch, O. (2004) Interspecies difference in cancer susceptibility and toxicity. *Pharmacol. Toxicol*. **31**: 917–970

Holtzman, S. G., Finn, I. B. (1988) Tolerance to behavioral effects of caffeine in rats. *Pharmacol. Biochem. Behav*. **29**: 411–418

Hopkins, S. J., Rothwell, N. J. (1995) Cytokines and the nervous system. 1: Expression and recognition. *Trends Neurosci*. **18**: 83–88

Kalmar, J. M., Cafarelli, E. (1999) Effects of caffeine on neuromuscular function. *J. Appl. Physiol*. **87**: 801–808

Khansari, D. N., Murgo, A. J., Faith, R. E. (1990) Effects of stress on the immune system. *Immunol. Today* **11**: 170–175

Lourdes, D. G., Manuel, R. M. (2001) Alterations induced by chronic stress in lymphocyte subsets of blood and primary and secondary immune organs of mice. *BMC Immunol*. **2**: 7–14

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem*. **193**: 265–275

MacCain, H. W., Bilotta, J., Lamster, I B. (1987) Endorphinergic modulation of immune function: potent action of the supeptide glycyl-L-glutamine. *Life Sci*. **41**: 169–176

Madden, K. S. (2003) Catecholamines, sympathetic innervation, and immunity. *Brain Behav. Immun*. **17**: S5–S10

Mandal, A., Batabyal, S. K., Poddar, M. K. (2007) Long-term caffeine-induced inhibition of EAC cell progression in relation to gonadal hormone status. *Ind. J. Exp. Biol*. **45**: 347–352

Mandal, D., Bhattacharyya, A., Lahiry, L. Choudhuri, T., Sa, G., Das, T. (2005) Failure in peripheral immunosurveillance due to thymic atrophy: importance of thymocyte maturation and apoptosis in adult tumor-bearer. *Life Sci*. **77**: 2703–2716

Modha, K., Whiteside, J. P., Spir, R. E. (1993) The determination of cellular viability of hybridoma cells in microtitre plate: a colorimetric assay based on neutral red. *Cytotechnology* **13**: 227–232

Mukhopadhyay, S., Poddar, M. K.(1998) Is GABA involved in the development of caffeine tolerance? *Neurochem. Res*. **23**: 63–68

Mukhopadhyay, S., Poddar, M. K. (2000) Long-term caffeine inhibits Ehrlich ascites carcinoma cell-induced induction of central GABAergic activity. *Neurochem. Res*. **25**: 1457–1463

Mukhopadhyay, S., Poddar, M. K. (2001) Caffeine inhibits the development of Ehrlich ascites carcinoma cell in female mice. *Ind. J. Exp. Biol*. **39**: 735–741

Mukhopadhyay, S., Mondal, A., Poddar, M. K. (2003) Chronic administration of caffeine: effect on the activities of hepatic antioxidant enzymes of Ehrlich ascites tumor-bearing mice. *Ind. J. Exp. Biol*. **41**: 283–289

Newell, M. K., Haughn, L. J., Maroun, C. R., Julius, M. H. (1996) Death of mature T cells by separate ligation of CD4 and T cell receptor for antigen. *Nature* **347**: 286–289

Oakley, R. H., Cidlowski, J. A. (1993) Homologous down-regulation of glucocorticoid receptor: the molecular machinery. *Crit. Rev. Eukaryot. Gene Expr*. **3**: 63–68

Ottaviani, E., Franceschi, C. (1996) The neuroimmunology of stress from invertebrates to man. *Prog. Neurobiol*. **48**: 421–440

Perandones, C. E., Illera, V. A., Peckham, D., Stunz, L. L., Ashman, R. F. (1993) Regulation of apoptosis in vitro in mature murine spleen T cells. *J. Immunol*. **151**: 3521–3529

Purves, H. D., Sirett, N. E. (1965) Assay of corticotrophin in dexamethasone-treated rats. *Endocrinology* **77**: 366–374

Ramanaviciene, A., Acaite, J., Ramanavicius, A. (2004) Chronic caffeine intake affects lysozyme activity and immune cells in mice. *J. Pharm. Pharmacol*. **56**: 671–676

Raubenheimer, P. J., Young, E. A., Andrew, R., Seckl, J. R. (2006) The role of corticosterone in human hypothalamic-pituitary-adrenal axis feedback. *Clin. Endocrinol*. **65**: 22–26

Ronson, A. (2006) Physiological stress in oncology: the role of glucocorticoids. *Bull. Cancer* **93**: 699–708

Russo-Marie, F. (1992) Macrophage and the glucocorticoids. *J. Neuroimmunol*. **40**: 281–286

Saino, N., Suffritti, C., Martinelli, R., Rubolini, D., Pape Møller, A. (2003) Immune response covaries with corticosterone plasma levels under experimentally stressful conditions in nestling barn swallows (Hirundo rustica). *Behav. Ecol*. **14**: 318–325

Sanders, V. M., Kohm, A. P. (2002) Sympathetic nervous system interaction with the immune system. *Int. Rev. Neurobiol*. **52**: 17–41

Schmitt, C. A. (2007) Cellular senescence and cancer treatment. *Biochim. Biophys. Acta* **1775**: 5–20

Seiki, K., Sakabe, K., Kawashima, I., Fujii-Hamamoto, H. (1990) Hormone and immune response, with special reference to steroid hormone, 1. A short review. *Tokai J. Exp. Clin. Med*. **15**: 91–99

Selmaj, K., Cross, A. H., Farooq, M., Brossnan, C. F., Raine, C. S. (1991) Non-specific oligodendrocyte cytotoxicity mediated by soluble products of activated T cell lines. *J. Neuroimmunol*. **35B**: 260–271

Severson, C. D., Burg, D. L. Larenz, D. E., Felsbush, T. L. (1987) An alternative method of panning for rat B lymphocyte. *Immunol. Lett*. **15**: 291–295

Shortman, K., Williams, N., Adams., P. (1972) The separation of different cells classes from different lymphoid organs. V. Procedures for the removal of cell debris, damaged cells and erythroid cells for lymphoid cell suspension. *J. Immunol. Methods* **1**: 273–287

Spindel, E., Arnold, M., Causack, G., Wurtman, R. S. (1980) Effects of caffeine on anterior pituitary and thyroid function. *J. Pharmacol*. **214**: 58–62

Spindel, E., Griffith, L., Wourtman, R. J. (1983) Neuroendocrine effects of caffeine. II. Effects on thyrotropin and corticosterone secretion. *J. Pharmacol. Exp. Ther*. **225**: 346–350

Straub, R. H., Cutolo, M. (2001) Involvement of hypothalamic-pituitary-adrenal/gonadal axis and peripheral nervous system in rheumatoid arthritis: view point based on a systemic pathogenic role. *Arthritis Rheum*. **44**: 493–507

Theiss, J. C., Shimkim, M. B. (1978) Inhibitory effect of caffeine on spontaneous and urethane-induced lung tumors in strain A mice. *Cancer Res*. **38**: 1757–1761

Thome, M., Tschopp, J. (2001) Regulation of lymphocyte population and death by FLIP. *Nature Rev. Immunol*. **1**: 50–58

Tranviverdi, F., Silveira, L. F., Mac-Coll, G. S., Bouloux, P. M. (2005) The hypothalamic-pituitary-gonadal axis: immune functions and autoimmunity. *J. Endocrinol*. **176**: 293–304

Turnbull, A. V., Rivier, C. L. (1999) Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. *Physiol. Rev*. **79**: 1–71

VanderPloeg, L. C., Welsch, C. W. (1991) Inhibition by caffeine of ovarian hormone-induced mammary gland tumorigenesis in female GR mice. *Cancer Lett*. **56**: 245–250

Visintainer, M. M., Volpicelli, J. R., Seligman, M. E. P. (1982) Escapable versus inescapable shock: differential effect on growth of implanted tumors. *Science* **16**: 437–439

Von Boehmer, H., Shortman, K. (1973) The separation of different cell classes from lymphoid organs IX. A simple and rapid method for removal of damaged cells from lymphoid cell suspensions. *J. Immunol. Methods* **2**: 293–301