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BETA-SITOSTEROL AND BETA-SITOSTEROL GLUCOSIDE STIMULATE HUMAN PERIPHERAL BLOOD LYMPHOCYTE PROLIFERATION: IMPLICATIONS FOR THEIR USE AS AN IMMUNOMODULATORY VITAMIN COMBINATION*

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Abstract—The phytosterols, ß-sitosterol (BSS), and its glucoside (BSSG) enhance the *in vitro* proliferative response of T-cells stimulated by sub-optimal concentrations of phytohaemagglutinin (PHA) several fold at extremely low concentrations (femtogram level). A 100:1 (mass:mass) ratio of BSS:BSSG (termed essential sterolin formulation, ESF) showed higher stimulation than the individual sterols at the same concentration. *In vivo* activity of ESF was also demonstrated when volunteers ingested ESF for 4 weeks. Proliferation of their T-cells, stimulated maximally with PHA, was significantly enhanced (20–920%) when compared to baseline values.

In vitro, ESF (1 μg/ml) was able to significantly enhance the expression of CD25 and HLA-Dr activation antigens on T-cells and increased the secretion, into the medium, of IL-2 and gamma interferon. NK-cell activity was also increased by BSS and BSSG alone, but with ESF a higher activity was always found at different effector:target ratios (100:1–12:1). © 1997 International Society for Immunopharmacology.

Keywords: beta-sitosterol, beta-sitosterol glucoside, lymphocyte proliferation, immunomodulation

INTRODUCTION

Selective modulation of the different components of the immune system has received considerable attention since it forms the basis for treatment of many pathological conditions such as organ rejection after transplantation, recovery from infectious diseases, treatment of cancer, auto-immune diseases and primary immunodeficiencies. According to Schmutzler *et al.* (1989) the term "immunomodulation" includes at least three therapeutic goals—suppression, stimulation and restoration. For suppression clinically effective drugs like corticosteroids and cyclosporin are available. For stimulation, however, clinical success has been achieved only with macromolecules like BCG and MDP (muramyl dipeptide) which serve as adjuvants to enhance B-cell activity. Levamisole is the only synthetic compound so far which was investigated as an immunostimulant on T-cell activity in controlled clinical trials (Renoux, 1980; Huskisson & Adams, 1980; Spreafico, 1980; Russell, 1980; Miller, 1980). Since its use was aimed at restoring a deficient immune system through non-antigen dependent stimulation, it can also be regarded as an immunorestorative agent.

St Georgiev (1988) has reviewed a relatively large number of synthetic chemicals with immunomodulatory or restorative properties. However, none of these has been developed to a stage of efficacious clinical use in humans. Wagner (1990) has reviewed plant derived natural products with immunostimulatory activity. They include alkaloids, quinones, terpenoids, phenolcarboxylic acids and high molecular mass compounds such as polysaccharides and glycoproteins. Of the latter arabinogalactans from *Echinacea* species have significant *in vitro* and *in vivo* immunostimulant properties.

One of our main research projects focuses on the development of anticancer drugs from plants. We

*The 100:1 ratio of BSS:BSSG is henceforth referred to as essential sterolin formulation (ESF).

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have recently investigated the *in vitro* cytotoxic action of the diglucoside hypoxoside extracted from *Hypoxis* species (Albrecht *et al.*, 1995a) and extended the study to a phase I clinical trial in which 24 lung cancer patients received a standardized *Hypoxis* plant extract (Smit *et al.*, 1995; Albrecht *et al.*, 1995b). The latter contained 50% hypoxoside, 10% beta-sitosterol (BSS) and 0.1% beta-sitosterolglucoside (BSSG). During the routine monitoring of the immune status of our patients we were intrigued by the finding that the lymphocyte proliferative responses towards phytohaemaglutinin (PHA) of some patients were elevated. Much to our surprise we found that BSS and BSSG were responsible for this phenomenon and that their action could be demonstrated *in vitro* at concentrations as low as femtograms per millilitre. When these findings are integrated with the excellent studies conducted by Salen *et al.* (1970) we came to the tempting conclusion that sitosterols have a vitamin-like action on the functioning of the immune system. Salen *et al.* (1970) have shown conclusively that, despite the relatively low absorption of BSS (5% or less of the daily intake), the plasma and faeces of patients on diets devoid of plant sterols rapidly became free of BSS and that its daily turnover equalled its daily absorption. It therefore implies that sitosterols are essential nutrients needed to be taken daily for the optimal functioning of the immune system.

In this paper we wish to report on the *in vitro* and *in vivo* effects of BSS and BSSG on human peripheral lymphocyte proliferation. We also demonstrate enhanced IL-2 and gamma interferon secretion and NK-cell activity by lymphocytes exposed to BSS and BSSG. Furthermore, we feel that although both sitosterols elicit these effects separately, a combination of the two, as it is found naturally in extracts of plants, is a more effective combination (Bouic & Albrecht, 1993).

Although the possible clinical applications of our findings are numerous we are of the opinion that the most acute of these will focus on the stabilization of the immune system of HIV positive patients.

EXPERIMENTAL PROCEDURES

Isolation of mononuclear cells and preparation of enriched T-cells

Venous blood, collected in preservative-free lithium heparin tubes was obtained from healthy laboratory personnel. The blood was diluted 50% with Tris-buffered RPMI 1640 medium and layered onto Lymphoprep. After centrifugation for 25 min at 400xg, the mononuclear cells were collected at the interface and washed 3 times with the Tris-RPMI by centrifugation.

T-cells were prepared from the mononuclear cell fraction by the rosetting method. Briefly, $3–5 \times 10^6$ cells, made up in RPMI + 10% fetal calf serum (FCS), were mixed with an equal volume of 1% sheep red blood cells (SRBC) treated with S-(2-aminoethyl) isothiuronium bromide hydrobromide (AET) and centrifuged to form a pellet. After overnight incubation at 4°C, the pellet was resuspended and separated by density centrifugation and the rosetting cells were recovered by lysis of the SRBC. These cells were used as an enriched source of T-cells.

In some experiments, total mononuclear cells were used in place of enriched T-cells and the enhanced proliferative response measured remained unchanged.

Assays for proliferative effects on T-cells

Proliferation of $1x10^5$ T-cells per well in 96 well plates was measured as ^{3}H-thymidine incorporation after 72 h of culture at 37°C and 5% CO_2 as previously described (Bouic *et al.*, 1989). As mitogen, PHA (phytohaemagglutinin, Wellcome) was used at sub-optimal concentrations as determined from a dose–response titration curve and all cultures were conducted in quadruplicate. The results were expressed as mean cpm ± SEM of quadruplicate determinations. The *in vitro* effects of BSS and/or BSSG were measured by adding the test compounds to the respective wells and comparison to wells having received mitogen alone. It should be noted that although sub-optimal PHA concentrations (1/5 maximum, i.e. 1 μg/ml) were used, similar (but less pronounced) enhancing effects of the sitosterol: sitosterol glucoside mixture were observed with optimal concentrations. It has also been suggested by other authors (Schmutzler *et al.*, 1989) that a given immunomodulator should be tested in the presence of sub-maximal antigenic stimulus.

BSS and the BSSG were obtained from Essential Sterolin Products, (Halfway House 1685, South Africa) and solubilized in 100% dimethylsulphoxide (DMSO) at 1 mg/ml concentrations. These stocks were then diluted in 100% DMSO and then 1:10 diluted in 100% FCS and again 1:10 directly into the wells of the culture plates. In so doing, the cells were in a medium containing 10% FCS and 1% DMSO final concentrations. Control experiments showed that 1% DMSO had no effect on the proliferative response of T-cells.

In vivo effects were measured on mononuclear cells obtained from volunteers who ingested BSS/BSSG or placebo. Ethical approval was obtained for the trial and Essential Sterolin Products provided capsules

containing 20 mg BSS and 0.2 mg BSSG, respectively. Placebo capsules were filled with talcum. The volunteers took one capsule 3 times daily. Blood was collected at 0, 2 and 4 weeks. The cells (1×10^5) were stimulated maximally with PHA and incubated with ^{3}H-thymidine for 72 h in order to compare their proliferative response with baseline values, i.e. before therapy was instituted.

Measurement of membrane activation antigen expression by flow cytometry

One million enriched T-cells were incubated for 24 h either in medium alone (unstimulated cells) or with PHA at sub-optimal concentrations (stimulated cells) as described above for the proliferative assays. ESF at a final concentration of 1 μg/ml was added to the test cultures at the start of the incubation. Following the incubation, the cells were washed and subsequently incubated with an aliquot of monoclonal antibody specific for the CD25 or the Class II Human Leukocyte antigen (HLA-Dr). These monoclonal antibodies were directly conjugated with fluorescein and were commercially available (Becton Dickinson, Bactlab Systems). After three final washes, the cells were analysed in a flow cytometer (FACScan, Becton Dickinson, Bactlab Systems) equipped with the analytical software programme Lysis II. The results were expressed as percentage of the cells positive for the antigen detected by the monoclonal antibodies.

Measurement of lymphokine secretion by T-cells

One million enriched T-cells were incubated with medium or PHA at sub-optimal concentration (as above) in the presence or absence of 1 μg/ml final concentrations of ESF. Forty-eight hours later, the culture tubes were centrifuged and the supernatants were analysed for their IL-2 and gamma interferon (INF) contents. A commercial kit was used for the determination of IL-2 content (range 0–60 IU/ml; British Biotechnology, Whitehead Scientific) while the gamma INF was measured by an ELISA method using recombinant antigen and monoclonal antibodies commercially available (Boerhinger Mannheim). This assay has a sensitivity of 1 IU/ml and a working range of 1–500 IU/ml.

NK-cell activity

The NK-cell activity was measured as the amount of ^{51}Cr released in the supernatant by the cancer cell line K562 as described by Ottenhof *et al.* (1981). Briefly, $2–5 \times 10^6$/0.1 ml K562 cells in RPMI + 10% FCS were pre-incubated with 100 μCi ^{51}Cr for 1.5 h at 37°C. These cells were washed several times and mixed with defined ratios of the effector mononuclear cells prepared from peripheral blood (effector:target ratios of 100:1; 50:1; 25:1; 12:1). After 4 h incubation, the plate was centrifuged and 100 μl of the supernatant counted in a gamma spectrometer. Spontaneous release and maximal release of the isotope were determined in the respective wells. The results were expressed as percentage specific lysis determined as:

$$\% \text{ Specific Lysis} = \frac{\text{Test cpm} - \text{Spontaneous cpm} \times 100}{\text{Maximum cpm} - \text{Spontaneous cpm}}.$$

The effector cells were incubated for 16 h prior to their addition in the assay with 1 μg/ml BSS and BSSG or ESF.

RESULTS

Enhancement of T-cell proliferation

An increase of approximately five-fold in ^{3}H-thymidine incorporation above suboptimal PHA stimulation was found when T-cells were incubated with 10 μg/ml BSS or BSSG. Without PHA addition the sitosterols were unable to stimulate thymidine incorporation. The enhanced proliferative responses found for enriched T-cells were not modified when total mononuclear cells (including B-cells and monocytes) were used. Furthermore, the addition of cholesterol (at equivalent concentrations) to the cell cultures did not result in enhanced responses of either enriched T-cells or total mononuclear cells (data not shown).

Figures 1 and 2 illustrate dose–response relationships in experiments designed to determine the minimum concentrations at which BSS and BSSG are able

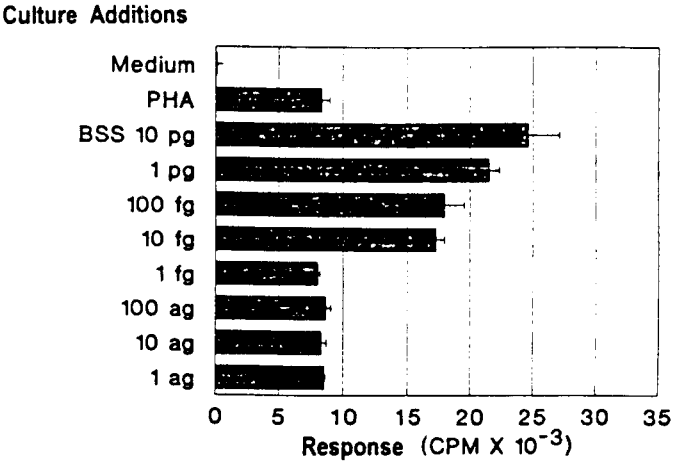

Fig. 1. ^{3}H-thymidine incorporation into T-cells stimulated suboptimally with PHA in the presence of different concentrations beta-sitosterol (BSS). See experimental procedures for details. The vertical bars represent the SEM of quadruplicate determinations.

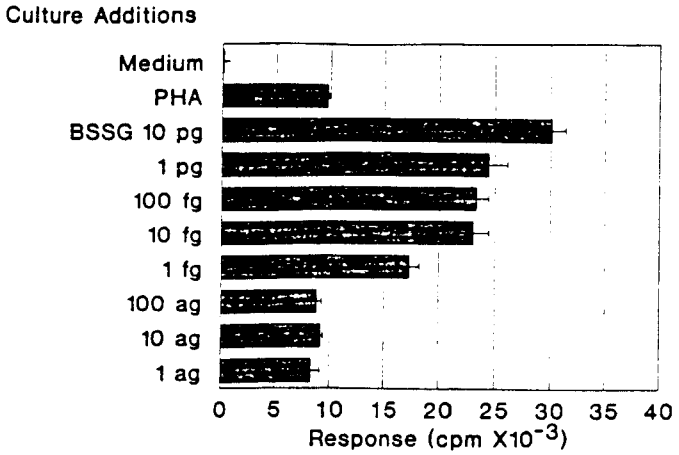

Fig. 2. ^{3}H-thymidine incorporation into T-cells stimulated suboptimally with PHA in the presence of different concentrations beta-sitosterol glucoside (BSSG). See experimental procedures for details. The vertical bars represent the SEM of quadruplicate determinations.

to illicit a stimulatory effect. From Fig. 1 it is clear that at a concentration as low as 10 fg BSS a significant stimulation (two-fold) is still exhibited. A comparable stimulation is shown for BSSG in Fig. 2, but at a ten-fold lower concentration (1 fg) than for BSS. In terms of number of sitosterol molecules per cell it can be calculated that at the lowest enhancing concentrations there are only 150 molecules BSS and 10 molecules BSSG present per cell. We have not measured endogenous BSS and BSSG concentrations in the FCS used in the incubations. However, it is likely that this source of sitosterol is mostly in the biologically inactive esterified form.

Figure 3 shows that when BSS and BSSG are mixed in a ratio of 100 BSS:1 BSSG (mass:mass) a larger

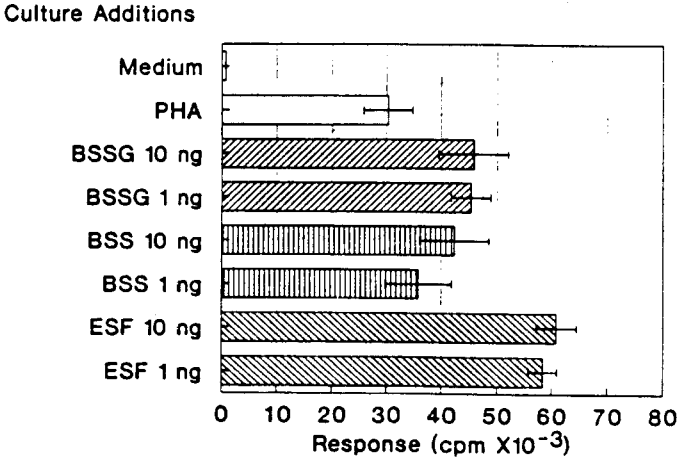

Fig. 3. ^{3}H-thymidine incorporation into T-cells stimulated suboptimally with PHA in the presence of different concentrations BSSG, BSS and ESF (100 BSS:1 BSSG). See experimental procedures for details. The vertical bars represent the SEM of quadruplicate determinations.

stimulation is obtained in comparison to the stimulation caused by the individual sitosterols at the same concentration.

Figure 4 shows that ingestion of the ESF by six volunteers for 4 weeks resulted in a statistically significant increase in the responses ranging from 20 to 920% at 4 weeks. In contrast the activity of the cells of two volunteers who took placebo remained unaltered over the same time period.

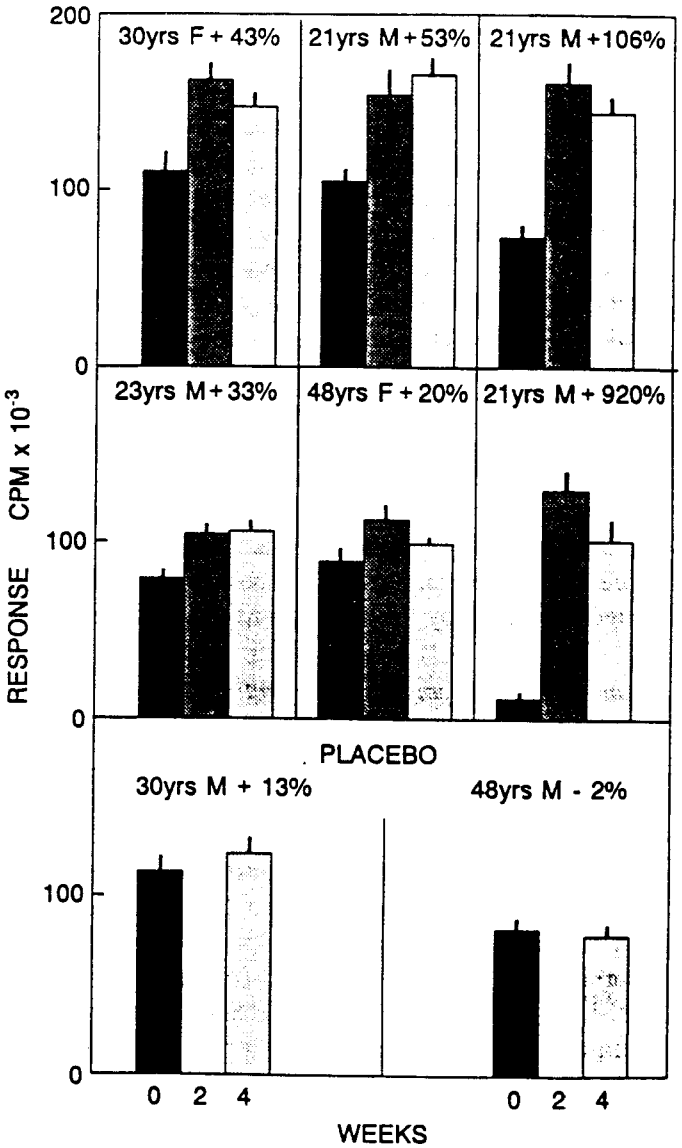

Fig. 4. T-cell proliferative response (measured as ^{3}H-thymidine incorporation) of volunteers who ingested one capsule t.i.d. of 20 mg BSS:0.2 mg BSSG (upper six graphs). The cells were stimulated maximally with PHA (see experimental procedures). The lower two graphs are for volunteers who ingested placebo capsules. The age (yrs), sex (male = M, female = F) and average percentage stimulation at 2 and 4 weeks relative to zero time are shown for each volunteer. The same batch of stock solutions was used for preparation of the cells and their proliferation after 72 h of incubation. The vertical bars represent the SEM of quadruplicate determinations at each time point.

Response–time relationship of in vitro T-cell stimulation

Since the enhancing effects of BSS and BSSG were being measured after 72 h of incubation, it was important to determine whether early activation events were being influenced or whether late events such as mitosis were being affected. For this, T-cells were incubated with the mitogen and at different times during the incubation period, the test compounds were added and the end point proliferative response was measured as usual. The results of a representative experiment are presented in Fig. 5.

As can be seen, the addition of BSS or BSSG to the cells at zero time results in the earlier demonstrated enhanced responses, but these potentiating effects become maximal once the cells have been pre-activated for 6 h. Thereafter, these effects decrease to original responses of the cells having received the mitogen only (control). These results suggest that the early activation signals involved in the proliferation of T-cells are being affected. Such events could include the acquisition of surface activation antigens (CD25 and HLA-Dr), and/or the upregulation of the genes for the synthesis and secretion of important factors involved in the growth and proliferation of T-cells.

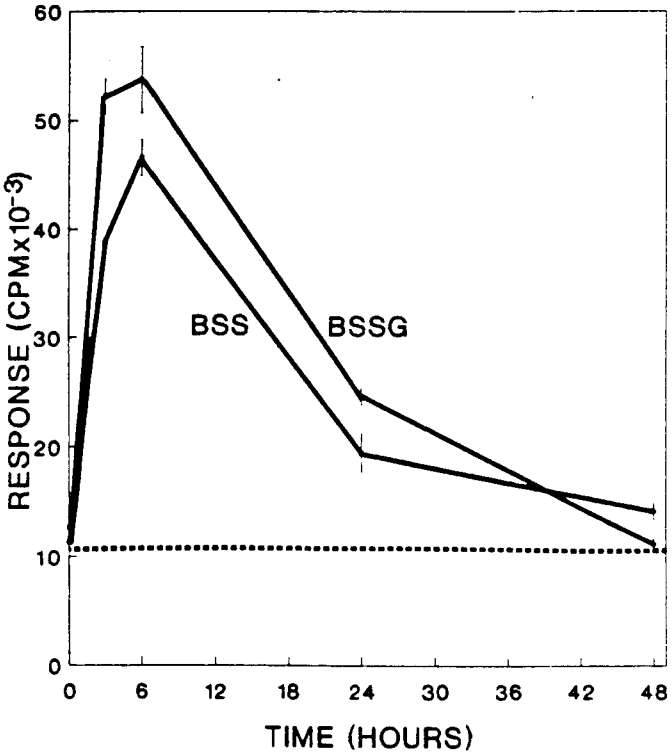

Fig. 5. ^{3}H-thymidine incorporation into 1×10^5 T-cells stimulated suboptimally with PHA and 10 fg/ml BSS or BSSG added at different times (0, 3, 6, 24 and 48 h) followed by a total incubation period of 72 h. The response of cells cultured in the absence of BSS or BSSG (controls) is depicted by the stippled line.

Effect of ESF on the expression of membrane activation antigens by T-cells in vitro

T-cells were incubated with suboptimal PHA and PHA plus 1 μg/ml ESF for 24 h and expression of activation antigens by the cells was then measured by monoclonal antibodies and flow cytometry. The results of a representative experiment are presented in Fig. 6.

As can be seen, PHA stimulated T-cells expressed both CD25 and HLA-Dr when compared to unstimulated cells. However, the addition of ESF results in approximately a doubling of expression of the same antigens. This indicates that the genes for the *de novo* expression of membrane antigens are up-regulated which possibly explains the enhanced proliferation of T-cells in the presence of BSS and BSSG as presented in Figs 1–3.

Effect of ESF on the secretion of IL-2 and gamma INF by T-cells in vitro

Table 1 shows the results from two representative experiments in which the co-culture of T-cells with PHA and 1 μg/ml ESF resulted in enhanced secretion of the lymphokines IL-2 and gamma INF. Indeed, stimulated cells released significantly more of the factors when compared to unstimulated cells. However, addition of ESF results in increases of 23% and 41% in the secretion of IL-2 and gamma INF, respectively, in experiment 1 and 17% and 36%, respectively, in experiment 2. Several repeat experiments have confirmed these observations.

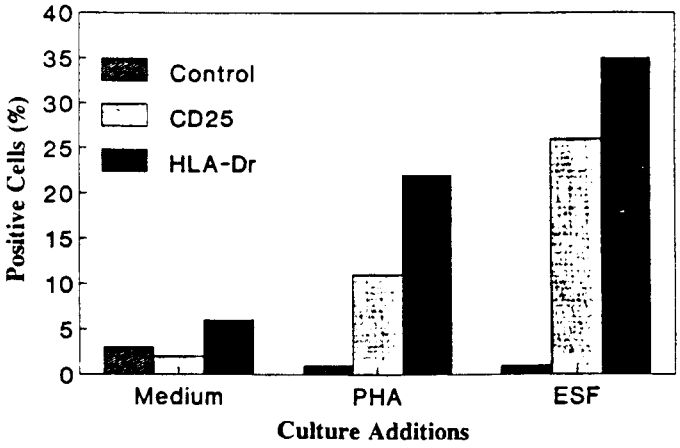

Fig. 6. The effect of 1 μg/ml ESF (100 BSS:1 BSSG) on the acquisition of activation antigens in T-cells (1×10^6) incubated for 24 h at suboptimal PHA concentration together with the relevant controls (PHA alone, medium alone). Percentage cells positive for CD-25 or HLA-Dr were determined by flow cytometry after binding of fluorescein conjugated monoclonal antibodies.

Table 1. The effect of 1 μg/ml ESF (100 BSS:1 BSSG) on IL-2 and gamma INF secretion by 1×10^6 T-cells suboptimally stimulated (or unstimulated) with PHA and incubated for 48 h. IL-2 and gamma INF concentrations were determined in the supernatants of the incubation medium by Elisa methods specific for lymphokines

	IL-2 (pg/ml)		Gamma INF (pg/ml)	
	Expt 1	Expt 2	Expt 1	Expt 2
Medium	0	9.8	54.4	79.4
PHA	363.8	145.8	214.0	182.0
PHA + ESF	447.8	170.9	302.0	246.7

Values represent the mean of duplicate determinations.

Effects of BSS, BSSG and ESF on the NK-cell activity in vitro

Figure 7 shows the increase in specific lysis of ^{51}Cr-loaded cancer cells incubated at different ratios with mononuclear cells activated with BSS, BSSG or ESF as described in the method section. It is clear that BSS and BSSG enhanced the lysis at all the different effector:target ratios, but that ESF was more effective in each case. These results are from a representative experiment. The trends of further repeat experiments were found to match exactly those shown in Fig. 7.

DISCUSSION

Approximately 80% of the total phytosterol content of higher plants is composed of BSS with about 1% in its glucosidated form (Akihisa *et al.*, 1991; Weihrauch & Gardner, 1978). BSS differs from the major animal sterol, cholesterol, only by an extra ethyl group in its side chain. Surprisingly, however, BSS shows several profound biological effects in a variety of experimental animal models. For instance, it has been shown that BSS can reduce carcinogen-induced cancer of the colon in rats (Barclay & Perdue, 1976; Hartwell & Abbott, 1969; Hartwell, 1976; Raicht *et al.*, 1980). It shows anti-inflammatory (Yamamoto *et al.*, 1991), anti-pyretic (Gupta *et al.*, 1980), anti-ulcer (Adami *et al.*, 1962; Xiao, 1992) and anti-complement (Yamada *et al.*, 1987) activity as well as insulin releasing (Ivorra *et al.*, 1988) and oestrogenic effects (Malini & Vanithakumari, 1991a, 1992) and inhibition of spermatogenesis (Malini & Vanithakumari, 1991b).

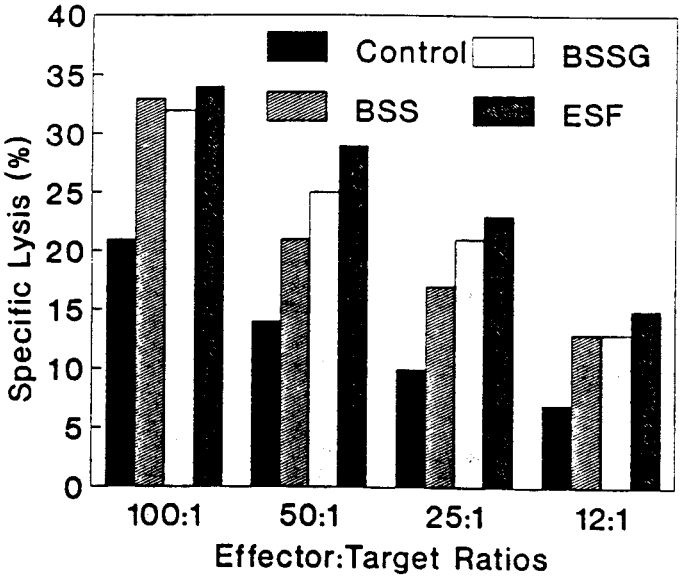

Fig. 7. The effect of 1 μg/ml BSS, BSSG and ESF (100 BSS:1 BSSG) on NK-cell activity as measured by % specific lysis of K562 ^{51}Cr-loaded cancer cells. The effector cells (T-cells) were preincubated for 16 h with the respective sitosterols before they were mixed with the target cells in the ratios indicated. Further details are provided in the experimental procedures section.

BSSG was recognised early in the century as a consistent obligatory component present in extracts of plants with medicinal properties (Power & Salway, 1913; Jantzen & Gohdes, 1934) and was, therefore, synthesized chemically (Salway, 1913). Most of the effects observed for BSS were also demonstrated for BSSG (Rios *et al.*, 1989; Gupta *et al.*, 1986; Seki *et al.*, 1985; Sugiyama & Seki, 1991).

BSS has so far been used in man for its cholesterol lowering effect (Salen *et al.*, 1970) and together with BSSG for treatment of benign prostate hypertrophy (Egghart & Gallyas, 1987; Pegel, 1984; Carbin *et al.*, 1990). There is, however, no doubt that the results presented here have wide-ranging implications for the further possible clinical use of BSS and BSSG in several diseases and pathological conditions. We showed that both BSS and BSSG, or a mixture (ESF) are capable of enhancing T-cell proliferative responses both *in vitro* and *in vivo* (Figs 1–4). We also showed that *in vitro* activated T-cells express more activation antigens and that they release more growth factors (IL-2 and gamma INF) in their supernatants (Fig. 6, Table 1). Furthermore, *in vitro* NK-cell activity is enhanced by BSS, BSSG and ESF. We also claim (Bouic & Albrecht, 1993) that a mixture of BSS and BSSG in a ratio 100:1 is more effective than either sterol alone (Figs 3 and 7).

Enthusiasm for the clinical use of BSS and BSSG has so far been dampened by the well known fact that the sitosterols are poorly absorbed from the gastrointestinal tract. In humans no more than 5% of the daily intake is absorbed (Salen *et al.*, 1970), while in beagle dogs a bioavailability of 9% was reported (Ritschel *et al.*, 1990). However, the fact that BSS and BSSG exert their effects *in vitro* on T-cells in hormonal concentrations (picograms to femtograms) is one argument against this paradigm. It was further shown that a genetic defect can cause excessive absorption

resulting in sitosterolemia (Bhattacharyya & Connor, 1974) which clearly indicates that the absorption of sitosterols from the gastrointestinal tract is a strictly controlled process. Finally, the studies of Salen *et al.* (1970) on BSS metabolism in man explain why it is physiologically "unnecessary" to absorb large quantities of sitosterols. They have compared the absorption and elimination kinetics of cholesterol and sitosterol in 12 patients and found the following:

1. Plasma concentrations of BSS ranged from 0.3 to 1.02 mg/100 ml for patients on a typical diet.
2. Plasma concentrations were raised little when daily intakes were increased greatly. However, on diets devoid of plant sterols, the plasma and faeces rapidly became free of BSS.
3. Like cholesterol, BSS is distributed in two pools (compartments) in the body. However, the size of the BSS pool measures only in milligram quantities as opposed to the gram quantities for the cholesterol pool.
4. Because there is no synthesis of BSS in the human body and its elimination rate is relatively fast, it is necessary to replace the daily loss by daily intake. On a "healthy" diet it means that the daily turnover of BSS is equal to its absorption. Likewise, insufficient dietary intake of sitosterols can rapidly result in deficient pool sizes which we theorize will result in deterioration of the functioning of the immune system. We believe that we have demonstrated this indirectly by the observation that the T-cell proliferative response of some of our volunteers increased by several hundred percent after additional intake of sitosterols (Fig. 4).

We are currently conducting clinical trials with ESF capsules in HIV positive patients since we are of the opinion that the *in vitro* effects described in this report would have beneficial effects in this pathology where gross immunological abnormalities have been reported. The results of these trials shall be published once available.

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