Pergamon

Int. J. Immunopharmac., Vol. 18, No. 12, pp. 693–700, 1996

Published by Elsevier Science Ltd. Printed in Great Britain
0192–0561/96 $15.00+ .00

PII: S0192–0561(96)00034–3

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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(*Received* 17 *August* 1995; *revised* 30 *April* 1996)

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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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.

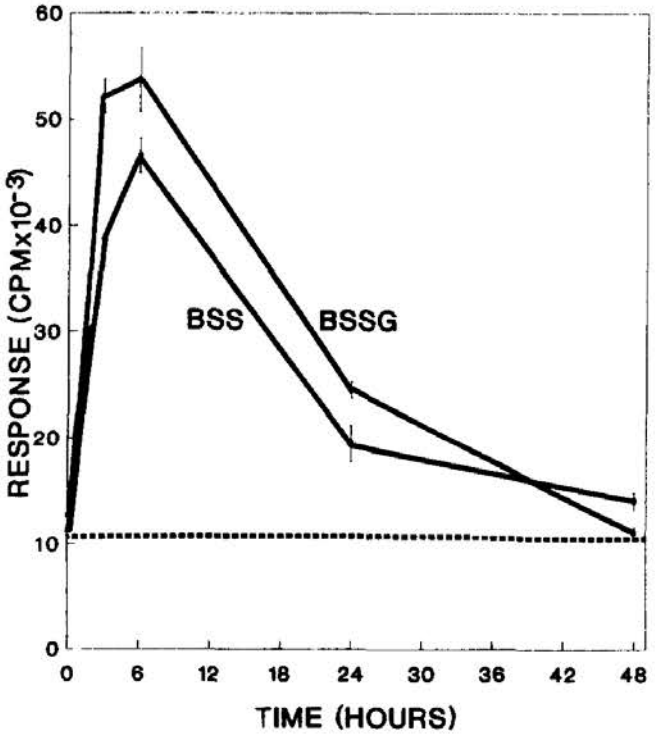

Fig. 5. ^{3}H-thymidine incorporation into 1×10^{5} T-cells stimulated suboptimally with PHA and 10 fg/ml BSS or BSSG added at different times (0, 3, 6, 24 and 48 h) followed by a total incubation period of 72 h. The response of cells cultured in the absence of BSS or BSSG (controls) is depicted by the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

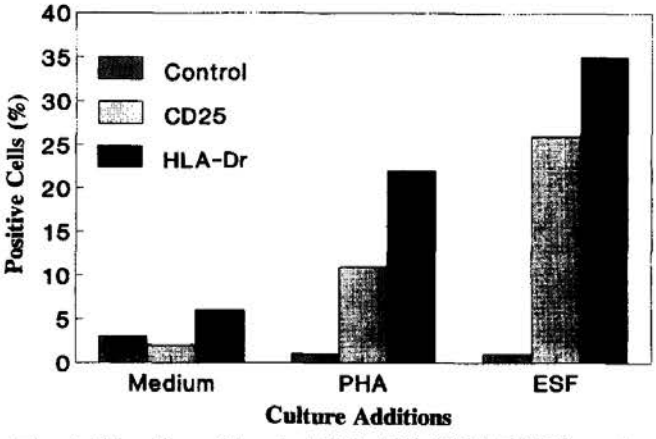

Fig. 6. The effect of 1 μg/ml ESF (100 BSS:1 BSSG) on the acquisition of activation antigens in T-cells (1×10^{6}) incubated for 24 h at suboptimal PHA concentration together with the relevant controls (PHA alone, medium alone). Percentage cells positive for CD-25 or HLA-Dr were determined by flow cytometry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

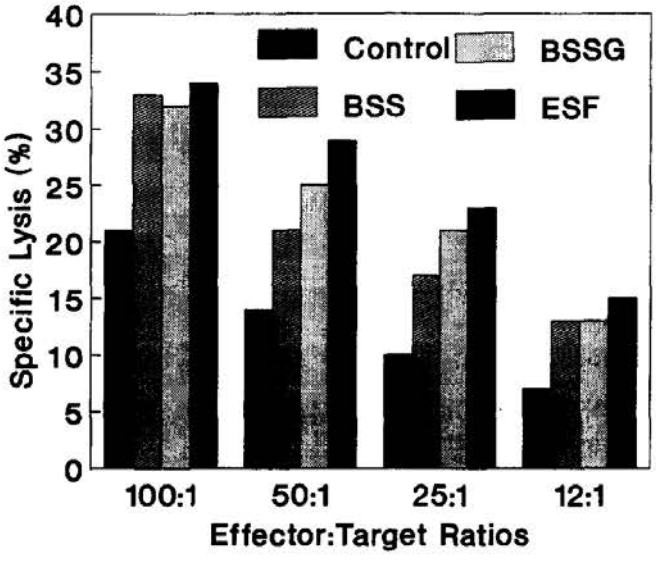

Fig. 7. The effect of 1 μg/ml BSS, BSSG and ESF (100 BSS:1 BSSG) on NK-cell activity as measured by % specific lysis of K562 ^{51}Cr-loaded cancer cells. The effector cells (T-cells) were preincubated for 16 h with the respective sitosterols before they were mixed with the target cells in the ratios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

Acknowledgements—This study was initiated and sponsored by Essential Sterolin Products according to an agreement entered into with the University of Stellenbosch.

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