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EVIDENCE THAT BISTRAMIDE A, FROM THE ASCIDIAN *LISSOCLINUM BISTRATUM* SLUITER, HAS IMMUNOMODULATING PROPERTIES *IN VITRO*

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ABSTRACT

We undertook a screening for agents affecting Concanavalin A (Con-A)-dependent and Lipopolysaccharide (LPS)-dependent proliferation of mouse splenocytes, from 632 extracts prepared from marine organisms collected in New-Caledonia. The aqueous extract from the ascidian *Lissoclinum bistratum* Sluiter was uniquely found to suppress Con A-dependent proliferation and stimulate LPS-dependent proliferation. Fractionation of the ascidian extracts revealed that such opposite effects on T cell and B cell proliferations can be exerted by a single component which was subsequently identified as Bistramide A (Bistratene A). This drug was previously isolated from the same organism for its cytotoxic properties toward various tumor cells. The therapeutic potentialities of both inhibiting T cell stimulation and stimulating B cell proliferation are discussed.

INTRODUCTION

During the last two decades considerable efforts were made to isolate compounds capable of modifying immunological reactions, in a pharmacological manner. Clinically important immunomodulating drugs were discovered on the basis of screenings from terrestrial or marine organisms (Bomford, 1988; Hadden, 1993; Werner, 1987). Thus, cyclosporine A, a cyclic undecapeptide from fungus, was found to inhibit the production of T cell-derived soluble mediators induced by antigens and lectins and hence to suppress the rejection of transplants. Additional clinically interesting agents with immunosuppressive properties such as FK506 (Kino et al., 1987), rapamycin (Seghal et al., 1975), and discodermolide (Longley et al., 1991) or immuno-stimulant activities like fungal glycans (Chihara et al., 1969) or muramyl dipeptides (Ellouz et al., 1974) were subsequently discovered, further stimulating the search for new compounds with higher immunoselectivity.

In the present paper we describe the result of a preliminary screening assay for agents affecting *in vitro* T cell stimulation and/or B cell proliferation, from 632 extracts prepared from marine organisms collected in New-Caledonia. We

found that extracts from the ascidian *Lissoclinum bistratum* Sluiter displays both suppressive and stimulating properties toward Con A-dependent and LPS-dependent splenocyte proliferation. A major compound exerting this striking dual effect was purified and identified as being identical to Bistramide A, a drug initially isolated from the same organism for cytotoxic properties (Gouiffes et al., 1988a).

MATERIALS AND METHODS

Animal Collection

All animals were collected by dredging down to 600 m depth or by scuba diving, within or at close proximity to the lagoon of New-Caledonia. The marine animals investigated in the present report belong to twelve categories. These are sponges, molluscs, sea cucumbers, ascidians, gorgonians, sea urchins, crinoïds, alcyonaria, ophiuroïds, madrepores, and two other groups of invertebrates. 354 marine species were collected and most of them immediately submitted to extract preparation, animals being put directly in fresh water on board. In some cases, however, animals were directly frozen on board at -30°C. Within 48 hours, frozen animals were crushed and put in water for the first extraction.

Preparation of Extracts

Three different extracts were prepared from each animal. The general procedure is indicated on Fig. 1.

Fractionation of Ascidian Extract by HPLC

Samples of *Lissoclinum bistratum* Sluiter were collected near UA islet in New-Caledonia, in February 1986, by divers of ORSTOM Nouméa. Specimens were identified by Dr. F. Monniot (Museum National d'Histoire Naturelle, Paris) who kept one sample. The aqueous extract was submitted to fractionation on high pressure liquid chromatography using a C_{18} column (Vydac) to which we applied a gradient of solvents from H_2O containing 0.1% Trifluoroacetic acid to a mixture of 40% H_2O, 60% acetonitrile and 0.1% Trifluoroacetic acid, in 60 min. The ultraviolet absorption was monitored at 230 nm. In analytical and preparative HPLC elution flow rates were 1 ml/mn and 3 ml/mn, respectively.

Assays with Mouse Lymphocytes

Fresh splenocytes ($2.5\ 10^6$ cells/ml) were taken from female Balb/c mice and suspended in a PRMI-1640 medium containing 1% non-essential amino acids, $5\ 10^{-5}$ M 2-mercaptoethanol, 4 mM glutamine, 2 mM sodium pyruvate, 10 mM HEPES buffer, 100 μg gentamicin and 1% fetal calf serum. The medium was then supplemented with either Concanavalin A (Con A, at 3 μg/ml as a final concentration) or Lipopolysaccharide (LPS from Sigma, at 3 μg/ml as a final concentration). The media were dispatched in the wells of flat-bottomed microplates (Nunc) in the presence of various samples (at 25 μg/ml as a final concentration) and then incubated for 2 days at 37°C in a humidified atmosphere containing 7% CO_2. ^{3}H-thymidine (1 μCi/well, 5 Ci/mmol, Amersham) was then introduced in each well and the cells were harvested 18 hours later. The radioactivity incorporated into splenocytes was determined, as previously described (Jackson and Bender, 1979). Each experiment was made in duplicate.

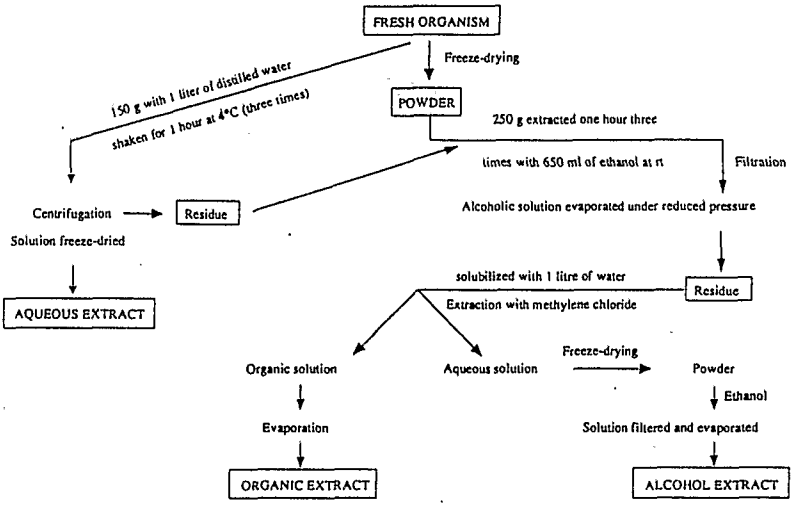


Figure 1. General procedure for extraction of marine animals; also used for *Lissoclinum bistratum*.

RESULTS AND DISCUSSION

In an effort to search for novel agents affecting the proliferation of immune cells, we prepared aqueous and alcoholic extracts from 354 marine species collected in New-Caledonia and investigated their ability to affect proliferation of mouse splenocytes. To tentatively identify the type of cells preominantly affected by our samples, the lymphocyte proliferation assays were performed in the presence of either Con A which essentially stimulates T lymphocytes or LPS which preferentially enhances B cell proliferation, both mitogenic agents acting through unknown mechanisms of action (Devlin and Hargrave, 1989). Approximately 20% of the investigated samples induced a clear immunostimulant (80 extracts) and/or immunosuppressive response (44 extracts) at a concentration of 25 μg/ml (dry weight) per assay. However, most of the samples displaying a strong immunosuppressive effect were also potently cytotoxic toward KB cells and P388 cells, thus offering an explanation as to the effects observed on splenocytes (C.D., unpublished results). Among all the samples affecting splenocytes proliferation, the aqueous extract from the ascidian *Lissoclinum bistratum* Sluiter displayed the interesting capacity of both suppressing the Con A-dependent splenocyte proliferation and increasing the LPS-dependent proliferation. This result suggested that the extract might either

contain both immunosuppressant and immunostimulant agents or a single agent with differentiate activities on T and B cells.

With the ultimate view to identify the compound(s) responsible for the observed effects, we submitted the aqueous extract from *Lissoclinum bistratum* Sluiter to a reverse phase HPLC on a C_{18} column using an appropriate solvent gradient (see Materials and Methods section). As shown in Fig. 2A, the crude extract contains a mixture of compounds. The fractions obtained were grouped in four pools numbered 1 to 4. Only pools 3 and 4 (Fig. 2B) retained apparent suppressive and stimulant effects on Con A-dependent and LPS-dependent splenocyte proliferations, respectively. To further characterize the activities of these two pools, we investigated their dose-dependent effects and compared them with that obtained with crude extract. As shown in Fig. 3, the Con A-dependent and LPS-dependent effects on splenocyte proliferation are differentially characterized in all cases. Thus, we observed that (1) suppression of the Con A-dependent effect occurs at lower concentrations compared to suppression of the LPS-dependent effects and (2) the LPS-dependent proliferation uniquely increased at low reagent concentration.

The most abundant compound in pool 4 (Fig. 2B) was further purified on HPLC as an apparently homogeneous compound called 4_A (Fig. 2C). Since a chemical work had been done previously on *Lissoclinum bistratum*, we have, thanks to the courtesy of Prof. J. F. Verbist, compared compound 4_A with some compounds isolated from this organism. Thus we found that compound 4_A is identical to Bistramide A (Fig. 2D) (Foster et al., 1992) by mass spectrometry (not shown); this agent was previously isolated from the same organism for its cytotoxic activity (Gouiffes et al., 1988b). Bistramide A was also shown to affect the protein phosphorylation patterns in HL 60 cells (Watters et al., 1992). As shown in Fig. 4,

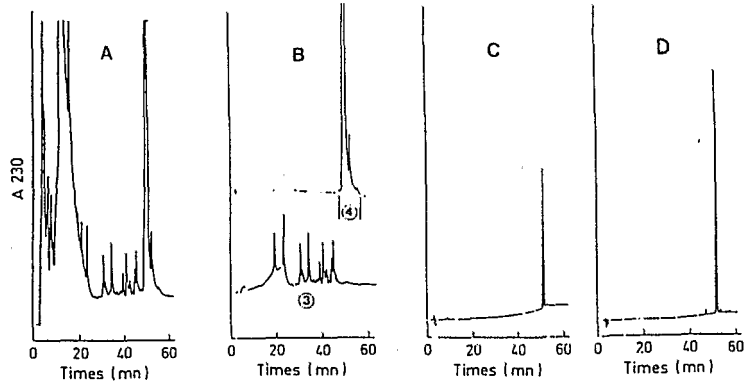


Figure 2. HPLC profiles of A) crude extract of *Lissoclinum bistratum*, B) fractions pooled in 3 and 4, C) compound 4_A, and D) bistramide A.

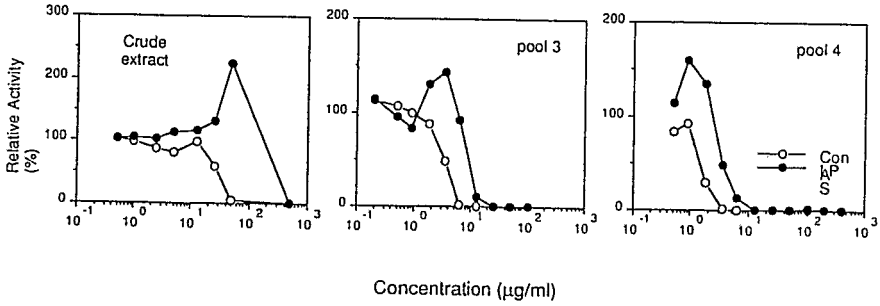
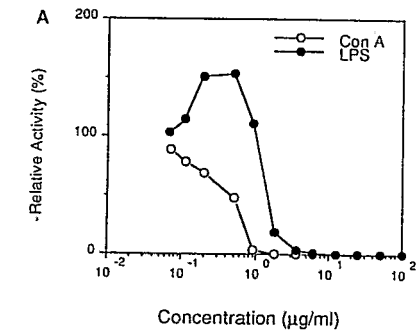


Figure 3. Immunosuppressive or immunostimulant effects of crude extract, Pools 3 and 4 of *Lissoclinum bistratum* on Con A and LPS mitogenic responses of murine splenocytes.



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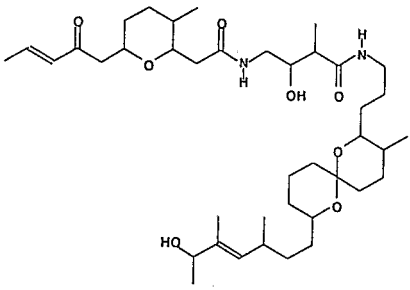

Figure 4. A) Immunosuppressive or immunostimulant effects of Bistramide A on Con-A and LPS mitogenic responses of murine splenocytes; B) Bistramide A.

Bistramide A affected splenocyte proliferations in a dose-dependent manner which is clearly identical to the effects of compound 4_A both in terms of concentration and amplitude. Probably, therefore, most of the immunoregulatory effects observed *in vitro* with pool 4 are due to Bistramide A. It is of interest that within a concentration ranging between 0.1 to 1 μg/ml (1.4 10^{-7} to 1.4 10^{-6} M), Bistramide A inhibits the Con A-dependent proliferation, whereas it increases the LPS-dependent proliferation. This differential behavior suggests, therefore, that Bistramide A has the dual ability of inhibiting T cell proliferation and of activating B cell proliferation. Above 1.4 10^{-6} M Bistramide A inhibits both the Con A-dependent and the LPS-dependent effects. Above this concentration, it could not be considered as being selective toward different cellular types. This result could be related to the cytotoxic effect of Bistramide A toward various tumor cells (Degnan et al., 1989). However, this immunosuppressive activity is unlikely to be due to cell membrane alteration as controlled by blue dye permeation test (data not shown).

We have shown that (1) an aqueous extract from the ascidian *Lissoclinum bistratum* Sluiter suppresses Con A-dependent and increases LPS-dependent splenocyte proliferation in a dose-dependent manner; (2) such a complex effect can also be assigned to Bistramide A; and (3) additional compounds such as those present in pool 3 might express similar properties. An agent capable of both suppressing T cell stimulation and activating B cell proliferation might be of considerable interest for controlling the regulation of an immune response. However, such a differential activity needs to be also observed under *in vivo* conditions. This aspect is now under investigation.

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