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Conference Paper

Immunostimulants of Plant Origin

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Our recent screening program with higher plants has revealed many low M_r compounds (*e.g.* alkaloids, quinones, terpenoids, phenolcarboxylic acids) and high M_r compounds (*e.g.* polysaccharides, glycoproteins) with a pronounced immunostimulating potential. The most promising low M_r compounds are some cytostatic compounds, which are known for their direct antitumoral activities at high doses and which exert immunostimulating effects when applied in minute doses. In the class of high M_r compounds, primarily some complex acidic arabinogalactans or rhamnogalacturonans (*e.g.* Echinacea purp., Achyrocline sat., Urtica dioica) show significant immunostimulating activities *in vitro* and *in vivo*.

INTRODUCTION

The term *immunostimulation* comprises a prophylactic or therapeutic concept aimed at stimulation of our *nonspecific* immune system. This primarily implies the non antigen dependent stimulation of the function and efficiency of granulocytes, macrophages, complement and natural killer cells. In contrast to immunity achieved by immunization or antibody injection, this type of immunity, arising from unspecific immunostimulation, is termed *paramunity*[1] and the agents responsible are known as paramunity inducers.

It is characteristic of these agents that they do not affect immunological memory cells. Their pharmacological efficacy fades quickly and must therefore be renewed by administering the drug either at intervals or continuously.

What do we expect from these kinds of drugs? Unspecific immunostimulation constitutes either an alternative or an adjuvant to conventional chemotherapy and prophylaxis of infections, for tumor- as well as autoimmune diseases, especially when the host's immune system is impaired.

Immunostimulation is also indicated for counteracting immunosuppressions or an ineffectively functioning immune system, manifested, for example, by a reduced resistance against infectious diseases, which may be a consequence of serious infections, physical and psychological stress, alcoholism, environmental damages such as pesticides, excessively applied chemotherapy, or long term treatment with immunosuppressive drugs. In undeveloped countries, malnutrition can be another cause of reduced resistance against infectious diseases.

DRUGS AVAILABLE TODAY

In the past, living and attenuated microorganisms, autologous and heterologous proteins and injections of animal organ preparations were used with the aim of restoring an impaired defence mechanism. At present, also thymus peptides and other Biological Response Modifiers (BRM) (*e.g.* interferons, interleukines), synthetic low molecular weight compounds (*e.g.* levamisol), chemically modified nucleotides, polysaccharides from fungi (*e.g.* lentinan) and, especially in Europe and China, some plant extracts are also used for the same purpose.[2]

The absence of any breakthrough in this area has various reasons:

- many preparations, *e.g.*, preparations from microorganisms and plants, are not chemically defined or are insufficiently standardized.
- the few synthetic compounds are too toxic.
- the externally administered BRM can exhibit severe side effects when applied in a too high concentration or at the wrong time since they have also important regulatory functions.
- furthermore, at the moment we are still unable to match the dose of an immunostimulant to the prevailing immune status and general constitution of a patient and in this way predict and optimize the efficiency in a proper way. Therefore, we need more basic research into the mechanism of action of the immunostimulants.

SELECTION OF DRUGS AND COMPOUNDS FOR SCREENING

In the search for plant constituents with immunostimulating potency, one can select those plant drugs which have been so far used in traditional medicine. You will not find the term immunostimulation in old literature. One can assume, however, that many plants described for their antibacterial, antiviral, antifungal or antitumoral activities are good candidates for screening.

TABLE I

Screening Methods for Detection of Immunostimulating (modulating) Compounds

In vitro phagocytosis a) microscopic smear test with human granulocytes b) chemoluminescence test with human granulocytes or macrophages c) flow cytometry	Leucocytes-migration inhibition-test Complement-tests (classical and alternative c.t.) Immune induced cytotoxicity tests (^{31}Cr or ^{3}H-Thymidin release from tumor cells or microorganisms)
In vivo phagocytosis on mice (Carbon clearance-test)	Interferon induction test
In vitro-T-lymphocyte transformation test a) with T/B-lymphocytes b) with subpopulations of T-lymphocytes (T_4/T_8 and NK-cells)	

Another criterium can be the case when the applied quantities of drugs with claimed antiinfectious or antitumour activities are so small that a direct antimicrobial or antitumour effect can be excluded and an immune induced effect can be assumed.

Meanwhile, a great number of compounds with potential immunostimulating activity have been described. In the first class of such compounds we find alcaloids, terpenoids, quinones as well as simple phenolic compounds, in the second class polysaccharides, peptides, glycoproteins and nucleotides (Ref. 2).

METHODS FOR SCREENING AND EFFICIENCY PROOF (see also Ref. 3)

For the screening of plant constituents, those *in vitro* and *in vivo* test systems are appropriate which allow determination of the functional state and the efficiency of the cellular and humoral unspecific immune system, in particular those which include granulocytes, macrophages, T-lymphocyte populations, NK-cells and the complement as target cells or systems.

The same test models can also be applied for preclinical trials. In addition, infectious stress (cytotoxicity) tests with *e.g. Candida albicans, Staphylococcus spp., Listeria enriettii* in mice or the tumor transplantation inhibition test have to be performed. In recent years, the flow cytometric methods have brought great methodological progress, especially for monitoring the immune status of a patient during immune therapy.[4]

RECENT RESULTS

a) Low molecular weight compounds (Figure 1)

The first chemically defined N-containing plant constituent that was found to exert immunostimulating activity was aristolochic acid.[5] In a double blind study, 0.9 mg/die administered orally over 21 days showed a significant enhancement of phagocytosis with a maximum on the 6th/8th day.

From the six oxindol alkaloids, isolated from the Peruvian plant *Uncaria tomentosa* (»Una de gato«), isopteropodin-HCl (**2**) was shown to be a powerful *in vitro* stimulant of phagocytosis in the concentration range of 10^{-3}–10^{-6} mg/ml.[6] Since the isomeric pteropodin and the other alkaloids were much less active or inactive, respectively, structure-activity relationships in the class of compounds must exist.

The observation that most of the investigated alkaloids (*e.g.* emetine, berberine, gelsemine) showed imuunosuppressive or cytotoxic effect when used at high doses and exhibited immunostimulatory properties only at very low doses has prompted us to investigate more closely this dose-dependent reversal effect. The naphthoquinone plumbagin (**3**) from *Plumbago zeylanicum* exerts *in vitro* immuno-suppressive or cytotoxic activity against primary cell cultures of granulocytes in a concentration range of 100 μg – 100 ng/ml, whereas it stimulates the same granulocytes in the extremely low concen-

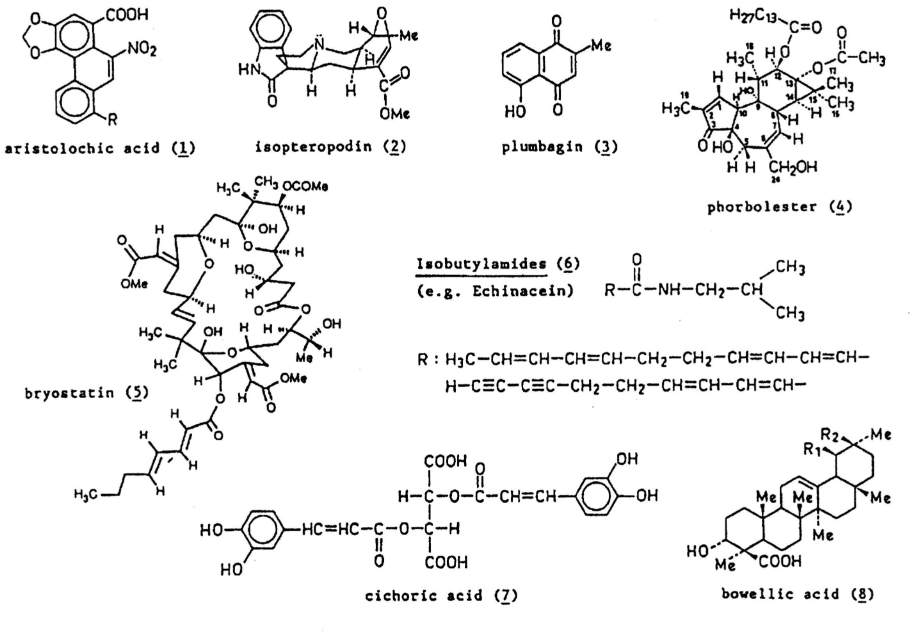

Figure 1. Plant derived natural products with immunostimulatory activity

tration range of 100 pg – fg*/ml.[7] Identical or similar effects on human granulocytes and T-lymphozytes were observed with other naturally occurring naphthoquinones (*e.g.* chimaphilin, alkannin, shikonin), with vincristin, colchicine, suramin, and the following synthetic drugs: azathioprine, cyclophosphamide, methotrexate and fluoroarucil.[7]

Since it has been reported that cells whose activities are suppressed are more sensitive than »noninfluenced cells«, we exposed lymphocytes to a brief cold treatment (cooling to 4 °C for 1 hr) and incubated them afterwards with vincristine, using the same concentration ranges. We found that the cold treatment resulted in a statistically significant increase in the stimulation rate (t-test 2.25; $p < 0.05$) which exceeds that of nonpretreated cells. The same effect was observed when the lymphocytes were pretreated at 40 °C for 30 min. After this finding, it was not surprising that phorbolesters (*e.g.* TPA) (**4**) and the recently isolated antitumour agents bryostatins (**5**) from the marine organism *Budula neritina*[8] also showed a similar dose dependant behaviour. Bryostatin I showed maximal stimulating effects on granulocytes and lymphocytes in the concentration range of 100 pg and 10 fg/ml, respectively. The results coincide with those of May *et al.*[9] who found that bryostatins, in addition to their known antineoplastic activities, show a multipotential stimulating effect in human haematopoietic progenitor cells. This again indicates that bryostatins can at the same time mimic many effects of the multipotential recombinant human granulocyte-macrophage colony-stimulating factor HGM-CSF.[10–12] In contrast to TPA, bryostatins lack a complete tumour-promoting ability.[13] The immunomodulating activity of cytostatic agents might result in another interesting effect on tumour growth. According to Sachs[14–16] various synthetic and microbial cytostatic agents (*e.g.* cytosin arabinoside, methotrexate or adriamycin) are able to achieve *in vitro* a differentiation of myeolotic leukemia cells and thereby a reversion of malignancy at doses of 3–7 ng/ml. Induction of differentiation was explained by the production of colony stimulating inducer proteins/MG T-2 as modulators or inhibitors of oncogene expression. When this triggering effect of cell differentiating processes by low doses of immunostimulatory agents can also be exhibited *in vivo*, a new promising concept of tumour therapy will be available.[17]

In the light of all these new findings, it is plausible to assume that many cancer drugs of plant origin, such as *e.g.* mistletoe (Viscum album), the South American lapacho (Tabebuia avellanedae), the extract of Dionaea muscipula and others, exert their antitumour activities by a total or partial immune induced mechanism of action.

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* 1 ng (nanogram) = 10–9 g; 1 pg (picogram) = 10^{-12} g;
1 fg (femtogram) = 10^{-15} g; 1 ag (attogram) = 10^{-18} g;

If we survey the vast number of plant drug constituents that may act as immunostimulators, we find also compounds which are neither irritants nor cytotoxic, in high doses, or carcinogenic. A detailed monitoring of *Echinacea spp.* extracts with several *in vitro* and *in vivo* immunological assays revealed isobutylamides (**6**), phenolcarboxylic acid esters (*i.e.* cichoric acid **7**)[18] and polysaccharides as the immunologically active principles of these drugs.[19]

The natural products described thus far can be classified as granulocyte, macrophage- and/or lymphocyte stimulators.

Since the human complement system also plays a very pronounced role in the immune defence system and in inflammation processes, we have also established an *in vitro* test system for screening isolated compounds for their complement activating effect on the classical and alternative cascade of the complement system.[20] Besides rosmarinic acid, some cinnamic acid derivatives and a few flavonol-acyl-glycosides as well as some triterpenoic acids were found to inhibit significally the classical way of complement cascade. (Table II).

TABLE II

Complement activating terpenoic acids

Structure	Inhibition of guinea pig complement at concentration*			
	0.1 mM	0.05 mM	0.01 mM	0.005 mM
boswellic acid (**8**)	80–90%	85–80%	40–50%	20–30%
crataegolic acid	100%	75–90%	10%	–
ursolic acid	100%	89–90%	<10%	–
glycyrrhetic acid	60–80%	20–30%	<10%	–
oleanolic acid	40–50%	10–20%	<10%	–

* mean values of 5 experiments

b) High molecular weight compounds (Figure 2)

In the last 20 years, a great number of water soluble polysaccharides with reported immunostimulating potential have been isolated from higher plants.[2, 21, 22] In Table III, the most intensively studied plants with respect to their polysaccharide composition are listed. The immunostimulating potential of these polysaccharides is described as »enhancing the phagocytosis of granulocytes and macrophages«, »inducing the Interferone-, Interleukine-, Tumor necrosis factor – production«, »complement activating« or »antitumoral«. The term »antitumoral«, first defined for several polysaccharides from fungi and algae, includes several kinds of interactions, in which macrophages as well as T-lymphocytes, NK-cells and their mediators can be in-

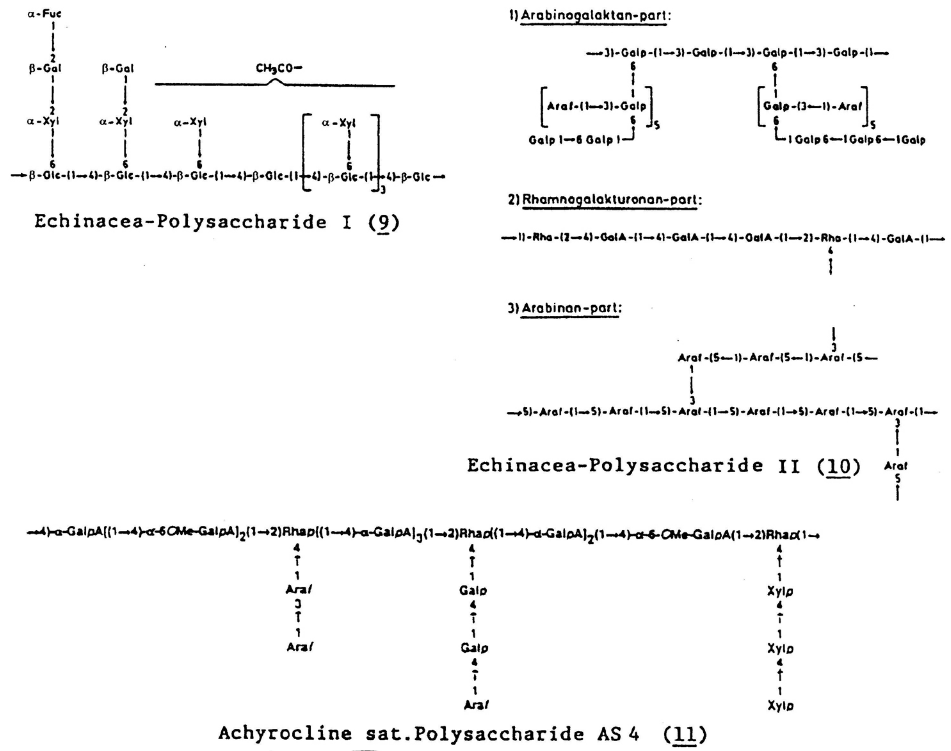

Figure 2

TABLE III

Immunologically thoroughly investigated polysaccharides from higher plants

Acanthopanax (Eleutherococcus) sent.	*Chamomilla recutita*
Achyrocline saturoioides	*Echinacea spp.*
Althaea officinalis	*Eupatorium cannabinum*
Angelica acutiloba	*Eupatorium perfoliatum*
Arnica officinalis	*Nerium oleander*
Astragalus mongholicus	*Panax ginseng*
Azadirachta indica, Sabal serrulata	*Solidago officinalis*
Baptista tinctoria	*Urtica dioica*
Bupleurum falcatum	*Viscum album*
Calendula officinalis	

volved. The expression »complement activating« can be understood or interpreted as an antigen processing mechanism or as an antiinflammatory activity because most of the complement tests measure the complement consumption only.

Since it is obvious that the various polysaccharides do not have the same points of attack in the immune system, it is at present hardly possible to draw a clear structure activity relationship and indicate which structural features are essential for an optimally immunostimulating activity. As far as the phagocytosis and macrophage stimulation are concerned, it is remarkable that neutral xyloglucans and glycuronic acid containing arabinogalactans or 4-0-methylglucuronoxylans predominate. Most of the active polysaccharides are highly branched with anionic structural units and M_r in the range of 20,000 to 50,000 and more. They derive from primary cell walls, have pectic or protopectic properties and some are viscous, belonging to the class of gums and mucilages.[21] In the class of potent complement activating polysaccharides, acidic polygalacturonan structures with arabinogalactan side chains are frequently found.[22]

It is assumed that the fine structures of these polysaccharides, *i.e.* tertiary structures, affect the mode of action.

As prototypes of both classes, the following polysaccharides are described in detail:

Echinacea species are the starting material of many phytopreparations in Germany and other European countries. The roots of *Echinacea purpurea* or *E. angustifolia* have been used by the North American Indians for wound healing and treatment of infections. Today, extracts of both plants are claimed to have immunostimulating activity and are used prophylactically and therapeutically as adjuvants for the management of infectious diseases and in particular for the treatment of chronic bronchitis, sinusitis and influenza. Besides some low molecular weight compounds (see first part), two chemically defined polysaccharides with immunostimulating activity have been isolated from the upper part of Echinacea purpurea.[19] Both compounds, a heteroxylan ($M_r \approx$ 35.000 D) and an acidic arabinorhamnogalaktan ($M_r \approx$ 450,000 D) stimulated granulocytes and macrophages and induced production of monokines (IL-1, LAF) in stimulated marrow macrophages and revealed high toxicity against tumour target cells, as measured by the ^{31}Cr-release assay.[23] Meanwhile, for a closer investigation, we were able to produce active polysaccharides also from plant cell cultures of Echinacea purpurea. From the extracellular polysaccharide mixture, after purification and separation by ethanol precipitation and column chromatography on DEAE-Sepharose Cl-6β, DEAE-Trisacryl M and Sephacryl S 400, three homogeneous polysaccharides could be isolated and structurally elucidated as two neutral fucogalactosylglucans ($M_r \approx$ 10,000 D and 25,000 D) (**9**) and an acidic arabinogalactan with the mean M_r of 75,000 D (**10**)[24] The polysaccharide with M_r 10,000 D was immunologically inactive. The other fucogalactosylglucans mainly stimulated the phagocytosis of granulocytes and macrophages. The acidic arabinogalactan was effective in activating macrophages to cytotoxicity against tumour cells[25] and *in vitro* as well as *in vivo* against microorganisms (*Leishmania enriettii, Candida albicans*). Furthermore, this polysaccharide induced macrophages to produce the tumour necrosis factor (TNF-α), interleukin –1(IL-1), interferon-β_2 and oxygen radicals, the latter measured by chemoluminescence. The arabinogalactan did not activate B-cells and did not induce T cells to produce interleukin-2, interferon-β_2 or interferon, but generated a slight increase of T-cell proliferation.[25] The great advantages of this polysaccharides are that it seems to act rather selectively on macrophages and that is is nontoxic over a wide dose range (acute toxicity > 4 g/kg i.p. or i.v.). Specific immune responses to red blood cells of sheep (antibody production) and to Listeria (DTH) were not affected by this polysaccharide. The positive infection stress tests performed with mice let us assume that this polysaccharide is a promising candidate for clinical trials.

The nearly exclusive activity of the Echinacea polysaccharide on granulocytes and macrophages is in some way unique, since *e.g.* an acidic arabinogalactan from mistletoe (*Viscum album*) of similar chemical composition does not enhance TNF from macrophages but strongly activates complement.[26]

In contrast to this compound, two further polysaccharides isolated from Achyrocline satureioides (Asteraceae), showed immunological activities in a great number of test models.[27] Both rhamnose, xylose and arabinose containing glykanogalakturonans ($M_r \approx$ 7,600, 15,000 D) (**11**) enhanced strongly the granulocyte and macrophage phagocytosis, showed a moderate effect on the TNF-α induction and exerted a strong anticomplementary activity in the classical as well as in the alternative pathway. It seems likely that these anticomplementary properties are responsible for the antiphlogistic activities of both polysaccharides as measured in the rat paw oedema model.[27] In comparison with indometacin (10 mg/kg) both polysaccharides exhibited the same activity (25–30% oedema reduction in 8 hrs) at concentration of 3 mg/kg at i.v. administration.

The same correlation between the anticomplementary *in vitro* effect and the antiphlogistic activity in experimental animals has also been found for a polysaccharide mixture from *Urtica dioica*.[28] If we compare our results on the anticomplementary activities of polysaccharides from higher plants with those of Ymada's group, we find a good congruence in that most of them are pectic heteroglycans with a relatively high proportion of galacturonan backbones. This finding agrees with our other finding that the most active anticomplementory polysaccharides from algae were found to be sulfated polysaccharides such as carrageenan, fucoidans, and heparin.[29] However, there are also neutral polysaccharides, *e.g.* Lentinan from the fungus *Lentinus edodes* and amylose, occurring widely in fruits and roots, which were found to be good anticomplementary active polysaccharides.[20]

In this context we should also mention the so called »antitumoral« polysaccharides from fungi, *e.g.* Lentinan, (*Lentinus edodes*), Schizophyllan (*Schizophyllum commune*) or Krestin (*Coriolus versicolor*). In most cases, these anitumour polysaccharides were shown to be glucans with different types of glycosidic linkages. Some structural features were obvious prerequisites: *i.e.* β-1 → 3-linkages in the main chain of the glucan core and further β-1, 6 branch points. Krestin is a β-1,4-glucan with β-1,6-glucopyranosidic side chains for evey forth glucose unit, containing a covalently bound peptide residue. The clinical usefulness of these polysaccharides, mainly in combination with chemotherapy, has been demonstrated with patients suffering from lung-, gastric-, colon- and cervical cancer.[30] I.m. injections once or twice per week with single doses, ranging from 2 mg to 30 mg of these polysaccharides, were generally shown to be effective in considerably prolonging the survival period of the patients.

From the *in vitro* and *in vivo* experiments performed so far, it can be concluded that these glucans exert their immune induced antitumour activities primarily by stimulating lymphocytes to liberate lymphokines (IL 2, MAF), thus activating NK-cells and macrophages.

As far a Krestin, as protein with covalently bound polysaccharide, is concerned, it is not clear whether, or to which extent, the protein part contributes to the immunological activity of this compound. On the other hand, there are a series of glycoproteins and proteins, such as lectins (*e.g.* concanavalin A), which exert modulatory influence within the immune system. The targets for these kinds of plant derived polymers are, at first hand, T-lymphocytes, NK-cells and the complement. *E.g.* it has been shown that non-toxic doses of the β-galactoside – specific lectin ML I from mistletoe (Viscum album) is able to activate tumoricidal effector mechanisms in experimental animals as well as in patients.[31]

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SAŽETAK

Imunostimulatori biljnog podrijetla

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U tijeku istraživanja višeg bilja pokazalo se da ono sadržava mnoge spojeve s izraženim imunostimulativnim potencijalom To su spojevi s niskim molekulskim masama (npr. alkaloidi, kinoni, terpenoidi, fenolkarboksilne kiseline) i spojevi s visokim molekulskim masama (npr. polisaharidi, glikoproteini). Među spojevima niske molekulske mase najperspektivnijima su se pokazali neki citostatici koji su u visokim dozama poznati po svojim antitumorskim djelovanjima, a u niskim dozama pokazuju

imunostimulativne učinke. U klasi spojeva visoke molekulske mase značajna imunostimulativna djelovanja *in vitro* i *in vivo* pokazuju u prvom redu neki kompleksni kiseli arabinogalaktani i ramnogalakturonani (npr. *Echinacea purp.*, *Achyrocline sat.*, *Urtica dioica*).