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From Hyperthermia to Immunomodulating Polysaccharides

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Abstract: Natural polysaccharides remain overlooked as part of naturally occurring bioactive molecules. The current knowledge results in numerous clinical trials, from first observations of their effects on various diseases to precise chemical characterization and decades of biological evaluation. The aim of this report is to summarize historical perspective of the route from the first anecdotical observation to the current position, with emphasis on some crucial turning points.

Keywords: Glucan, History, Thermotherapy, Zymosan, Cancer

Introduction

The title is not as straightforward as indicated, however, certain relations existed and exist so far and this review strives to mention them. Hyperthermia can be induced by externally brought heat (it is sometimes called thermotherapy) or for curative purposes, artificially provoked "true" fever caused by infection; the second case is denoted pyrotherapy. While the classical pyrotherapy, whose application culminated in the first half of the last century, is used only rarely for it can bring many negative consequences, certain kinds of thermotherapy, such as contact heating, shortwave diathermy, infrared heating, ultraviolet therapy, microwave thermotherapy, paraffin baths, *etc*., are in indicated cases used routinely.

The curative effects of elevated body temperature have been known since long ago. In ancient times there was a saying, “Give me the power to produce fever and I'll cure all diseases,” attributed alternately to Hippocrates of Kos, Parmenides of Elea or Galen of Pergamum. Hippocrates wrote about the beneficial effects of fever to epilepsy and Galen described curing a melancholic patient after the attack of quartan malaria (Coxe, 1846). Even in the modern age using therapy with increased temperature experienced considerable curative achievements.

A historic example of thermotherapy was the treatment of syphilis, which epidemically spread in Europe in the 15th century and with the high virulence and very pernicious symptoms afflicted populations of all social classes; the epidemic spreading of syphilis was supported by mass movement of army troops as well as civilians. It is very probable that the infection was brought to Europe by Columbian sailors from the Caribbean, where treponematoses are endemic (Rothschild, 2005). Besides mercury preparations, obligatory used for the treatment any skin conditions, or a guaiac wood extract, during those times sudatory baths were used for syphilis treatment (Paracelsus, 1530). These procedures represented a really torturing method, a more modest way consisted of closing a patient in an overheated room for several hours or days, in the rougher manner, dropping him into steaming horse manure. Results of these methods, taking advantage of thermosensitivity of spirochaetes, were uncertain since for the total eradication of the infection rather high temperature is necessary and on top of that, not everyone survives such a procedure.

Pyrotherapy in Modern Medicine

Application of pyrotherapy in modern medicine is dated to the end of 19th century. In this time, many cases were described about amelioration of paralytics after infection of feverish illnesses, *e.g*., pneumonia, erysipelas, typhus, *etc.* This information inspired an Austrian psychiatrist Julius Wagner-Jauregg to experiment with different pyrogens to treat general paresis, non-curable and perniciuos during these times. From a series of tested pyrogens, among others tuberculin, infection of *Spirillum minus* or erysipelas streptococci, *etc*., the best results were obtained by the infection of malarial plasmodia (Wagner-Jauregg, 1922). Using combination with other customary used treponemocidal drugs (Salvarsan, compounds of iodine

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or bismuth), remissions even total recovery were achieved; as matter of course, after the therapy it was necessary to cure the malaria. Wagner-Jauregg was awarded by Nobel Prize for medicine in 1927 for these methods. In the 40's of the last century this somewhat dangerous thermotherapy was substituted by penicillin and later by other antibiotics, but was probably still used in 70's (Heimlich, 1993).

In the 19th century it was also known that certain infectious diseases have beneficial therapeutic effects on malignant proliferation. This effect was also ascribed to accompanying temperature elevation; today, of course, it is known that it is predominantly the consequence of immunopotentiating effects of the infection resulting in changes of homeostasis mediators. The first purposed utilization of this effect is dated to the 50's of the 19th century, when Busch (1968) tried to utilize fever, caused by the infection of erysipelas, so by *β*-hemolytic streptococci of group A, as treatment for the sarcoma localized in the soft tissue of a patient. Really, time-limited remission occurred, but the total course of the ailment was not managed. Three decades later the use of erysipelas for curing of pernicious tumors was studied more steadily by William B. Coley, probably having not known the pioneer work of Busch (Coley, 1891). First, Coley used injections of live cultures of *Streptococcus pyogenes* (Bickels *et al*., 2002; Coley, 1893), but in this insecure method he later replaced live cultures with heat inactivated ones. As it was known that virulence of the streptococci increased at the presence of *Serratia marcescens* (in those times this red-pigmented microbe was called *Bacillus prodigiosus*) he combined streptococci with this microbes; the combination of cultures of these two microbes, inactivated by heating to 75°C, is known as the Coley's toxin or the Coley's vaccine (Coley, 1894). Application of the Coley's toxin leads to violent increasing of the body temperature to 40-40.5°C. Coley applied his toxin-more or less successfully to inoperable sarcomas of the soft tissues (Coley, 1910; 1928), but also to metastases to the long bones (Coley, 1913). It is interesting that at present there are attempts made to revive the application of Coley's toxin to tumor treatment (Maletzki *et al*., 2012), despite too risky combination of dangerous pathogens-streptococcal infections are well known and also *Serratia* is not an innocent microbe, but an opportunistic pathogen, the agent of torpid nosocomial infections.

Lipopolysaccharide

The presence of Gram-negative *Serratia* led to supposition that the active pyrogenic agent in the Coley's should be lipopolysaccharides (LPS, endotoxins, toxic polysaccharides), which are the typical component of all Gram-negative microorganisms. As was found later, this supposition was not correct, for the pyrogenicity was not the main curative effect and beneficial outcomes of Coley's toxin relate to function manifestation of interleukin IL-12 (Tsung and Norton, 2006) or tumoricidal activities of pigments prodigiosins (Chang *et al*., 2011; Stankovic *et al*., 2014; Darshan and Manonmani, 2015). However, during past times the mentioned supposition led to an attempt to replace the arguable intact microbes, though inactivated, by isolated and purified lipopolysaccharides.

Lipopolysaccharides are contained in outer membranes of all *Enterobacteriaceae* and most of the other Gram-negative microbes; LPS represents here up to 75% of the cell surface (Molinaro *et al*., 2002). General scheme of the LPS structure is preserved at all Gram-negative microorganisms, but in details it can dramatically differ. At different microorganisms and even at serotypes of the same microbe substantial differences can be found first of all in the polysaccharide moiety. At the external end of a lipopolysaccharide complex polysaccharide with antigen properties (*O*-antigen, OPS) is bound, having up to 40 monosaccharide units. This long polysaccharide is linked to several molecules of fatty acids (antigen *A*) through the central oligosaccharide core, 3-deoxy-D-mano-oct-2-ulosonic acid (Kdo) (Molinaro *et al*., 2002; 2015) and glucosaminyl-*β*(1-6)-D-glucosamine, usually fosforylated in positions 1 and 4. The fatty acids are anchored in the outer cell membrane and form its constituent part. In the *O*-antigens of different microbial origin more than 60 monosaccharidic and about 30 non-saccharidic moieties were detected (Raetz and Whitfield, 2002). The fatty acids usually contain 12-16 carbon atoms and tend to be hydroxylated on the third carbon atom (Raetz, 1990).

One of the first research studies of the medicinal application of isolated lipopolysaccharides was Shear, who worked with purified LPSs from *Serratia marcescens* and *Escherichia coli.* Shear published several papers (see for example citations (Shear and Turner, 1943; Shear, 1944); from these times there were published hundreds of papers dealing with physiological effects of LPSs from different sources.

In mammals, lipopolysaccharides act as a considerable stimulator of the innate immune system through the mediation of TLR4 (Poltorak *et al*., 1998; Kalis *et al*., 2003) or, as was recently found, even of intracellular receptors (Kayagaki *et al*., 2013) activating caspase-11 (Hagar *et al*., 2013). When LPSs are applied parenterally, considerable hyperthermia occurs, accompanied by a strong inflammatory response leading to increasing resistance against infection, as well as to regression of tumors via activation of TNF; the hyperthermia itself is only a concomitant reaction and does not represent the essence of the LPSs impact.

Massive immune response, however, induces very unfavorable side effects, such as diarrhea, leucopenia, hypotensive shock, intravascular coagulation and in the worst cases septic shock and Multiple Organ Dysfunction Syndrome (MODS). The curative effect of LPSs is then considerably doubtful. Even if we could to eliminate these side effects by careful dosage, during protracted treatment LPSs lose efficacy due to quickly developing tolerance (Zeissberger and Roth, 1998; Cavaillon *et al*., 2003).

Many authors studied composition as well as toxicity of lipopolysaccharides (see reviews (Nowotny, 1963; Lüderitz *et al*., 1966; Raetz, 1990); probably the most complete is the review of Nowotny (1969). For the subsequent development it is important that as the toxic principle of LPS was identified lipid A, the true endotoxin (*e.g*., Gardner *et al*., 1939; Westphal and Luderitz, 1954) whilst the polysaccharide subunit (*O*-antigen) acted as an antigen determinant; when *O*-antigen is missing, antigen properties are determined by the oligosaccharide core. The primary role of the polysaccharide subunit is protective and the unit is practically non-toxic (Raetz and Whitfield, 2002).

These findings logically started the investigation of different bacterial polysaccharides, not only those contained in LPS. So for example, in the 40's of the last century, Shear and coworkers isolated from the culture of *Serratia marcescens* a substance, which caused necrosis of tumors, as mentioned above (Shear and Turner, 1943). Lately, Srivastava and Adams (1962) found that it was a mix of three different polysaccharides with similar chemical structure. Their main chain contains D-glucose and D-mannose units linked by (1-3)-glycosidic bonds. Some glucose units are branched on C-2 and C-4 and polysaccharides differ in the degree of that branching and also in the ratio of both sugar components.

Zymosan

In regard to justified distrust of bacterial sources of polysaccharides, attention gradually shifts to much less dangerous microorganisms, first of all to yeasts. Their intact cells could be, of course, also pyrogenic (Kobayashi and Friedman, 1964) and pyrogenic was also the first isolate from them-zymosan (Hinz *et al*., 1961), which will be mentioned below. Of course, this pyrogenicity, given mostly by contaminating proteins, is negligible compared to the other effects. Pure isolated polysaccharides are apyrogenic and their biological effects have nothing to do with it.

In the 40's of the last century, Pillemer and Ecker isolated by the alkaline extraction of intact cells of *Saccharomyces cerevisiae* an insoluble fraction (Pillemer and Ecker, 1941), which inhibited the third component of complement (C3). This preparation was named zymin or Ecker fraction and lately zymosan; a mention of inactivation of complement by yeast cells is substantially older (von Dungern, 1900), though. Coincidentally, at the same time Pillemer and coworkers isolated protein from the human serum as well as sera of different mammals, which destructed bacteria and neutralized viruses by activation of complement without participation of antibodies. They named the protein properdin (from Latin *perdere*, to destroy, kill) and described its role in the immune system in a series of papers (the first was published in 1954 (Pillemer *et al*., 1954), the last one in 1957 (Pillemer *et al*., 1957). In these papers they mentioned the effect of zymosan on the level of properdin in the serum. In the course of time, the "properdine system" was refused and at last rehabilitated (Kemper and Hourcade, 2008; Harboe *et al*., 2012), but did not influence the fate of zymosan, which began to be applied in many other cases; today zymosan is even commercially available. Zymosan is primarily a potent stimulator of alveolar macrophages and among others induces the release of a series of cytokines, first of all IL-8, from human neutrophils.

β-Glucan

Even though zymosan was able to stimulate non-specific immune response, it was not clear which of its components was responsible for that activity. Actually, zymosan is not a pure polysaccharide but rather a crude insoluble product which contains about 50% of β-glucan and also around 17% of mannan, 14% of a protein and some ballast substances (DiCarlo and Fiore, 1958). Detailed investigation led to the conclusion that the active component, responsible for the primary effect of zymosan, is branched polysaccharide *β*(1-3),*β*(1-6)-D-glucan (Riggi and DiLuzio, 1961; Pontieri *et al*., 1963; Czop and Austen, 1985; Williams *et al*., 1986) (hence shortly: β-glucan). Probably the first attempt to compare physiological effects of pure β-glucan and zymosan was done by a team from Tulane University in New Orleans, headed by Nicholas DiLuzio *et al*. (1970). This polysaccharide forms a substantial part of the cell walls of yeast and also many micro- or macromycetes (Table 1). From the point of view of immunological functions it is important that β-glucan, except for some harmless yeasts, is also an obligate component of the cell wall of human pathogenic fungi, such as *Candida albicans, Aspergillus fumigatus, Cryptococcus neoformans*, *Histoplasma capsulatum, Coccidioides immitis* and others. This is the main reason immune reactions trigger after contact of β-glucan with the proper receptor on certain immune cells (macrophages, monocytes, dendritic cells, neutrophils).

Table 1. Composition of cell wall polysaccharides of some "fungi"

Kingdom	Division	Class	Order	Genus and Species	Prevailing wall polysaccharides at vegetative cells[a]
Protista	Myxomycota	Acrasiomycetes	Dictyosteliales	*Dictyostelium discoideum*	Cellulose, glycogen
Chromista	Ooomycota	Oomycetes	Peronosporales	*Plasmopara viticola*	Cellulose, β-glucan
				Phytophora infestans	
		Hyphochytridiomycetes	Hypochytriales	*Rhizidiomyces parasiticus*	Cellulose, chitin
Fungi	Chytridiomycota	Chytridiomycetes	Blastocladiales	*Blastocladiella emersonii*	chitin, β-glucan
	Eumycota	Zygomycetes	Mucorales	*Mucor mucedo*	chitin, chitosan
		Ascomycetes	Eurotiales	*Aspergillus fumigatus*	chitin, β-glucan
		Hemiascomycetes	Saccharomycetales	*Saccharomyces cerevisiae*	β-glucan, mannan
				Candida albicans	mannan
	Ascomycota	Eurotiomycetes	Onygenales	*Coccidioides immitis*	β-glucan, chitin, mannans
				Histoplasma capsulatum	β-glucan, α-glucan, chitin
				Blastomyces dermatitidis	
				Paracoccidioides brasiliensis	

[a]Composition of the cell wall of yeast-like and filamentous forms of dimorphic fungi can be different

There are various natural sources of β-glucans it can be isolated from yeast, mushrooms, seaweed, bacteria, various grains and even protozoa. However, there are three main sources: Mushrooms, yeast and grain. The reasons are mainly historical the Western civilization has consumed both bread and beer for centuries, therefore there is a significant surplus of yeast. Similarly, the Far East is known for adding mushrooms to the regular diet and various mushrooms are part of the old folk remedies. Grain glucans are the result of a surplus of various grains in Canada and Australia.

It is necessary to mention another way leading to information about specific physiological properties of isolated *β*-glucan. Studies of remedial character of medicinal Asian fungi (shitake-*Lentinula* (*Lentinus*) *edodes,* maitake-*Grifola frondosa*, hiratake-*Pleurotus ostreatus* and some others) showed that these fungi, besides other physiologically active compounds, contain mainly β-glucan. Pioneering work done by Goro Chihara from the National Cancer Center in Tokyo, who isolated it from *Lentinula edodes*, named such isolate lentinan (Chihara *et al*., 1969). In the course of time, both of the ways mentioned gradually converted. First reports, however, can be traced to the study of Ringler and Lukas *et al*. (1957). Decades of research revealed several types of action. The first level of action is active in the early stages of carcinogenesis and involves enhancement of several facets of immune response, including IL-1, IFN-γ, TNF-α and IL-12 production, NK cell activity and macrophage activation (Vetvicka and Vetvickova, 2015). The role in NK cell reconstruction during cancer treatment seems to be particularly important (Pohorska *et al*., 2016). Another level of protection involves an increase of antioxidant capacity and detoxification of mutagenic compounds (Fujimiya *et al*., 1988).

The third, clinically possibly the most relevant, effects involved cooperation with anti-tumor antibodies. *In vitro* and *in vivo* data indicated that a combination of antibodies and glucan offers superior effects (Liu *et al*., 2009a), making it an extremely important finding. Normally, the majority of malignant cells in solid tumors are naturally targeted with C3 fragment. Freshly excised tumors and established cancer cell lines were examined for tumor opsonization and natural antibodies and the opsonization with circulating anti-tumor antibodies and C3 was confirmed (Niculescu *et al*., 1992).

Humanized anti-cancer monoclonal antibodies (such as Herception Avastin, Rituximab or Erbitux) are currently widely used to treat tumors (Adams and Wiener, 2005), despite often questionable results. One possible solution is to use these antibodies in combination with common chemotherapy, often increasing adverse side effects. Studies showing that yeast-derived glucans can augment anti-tumor antibody efficacy to treat cancer (Hong *et al*., 2003; 2004; Yan *et al*., 1999) were confirmed using barley-derived glucan leading to clinical investigation in Phase II trials (for review see Liu *et al*. (2009b). As these effects cannot be reproduced in C3- or CR3-deficient mice, the importance of CR3 and C3 is clear (Yan *et al*., 1999). As phagocytosis of glucan does not require CR3, this uptake suggested that in addition to CR3, other glucan receptors, including Dectin-1, have to participate.

Despite decades of research and numerous clinical trials, anti-tumor effects of glucans and the underlying mechanisms are still not fully understood. Some studies showed that besides direct stimulation of immune reactions, up-regulated expression of the tumor suppressor gene p53, cell cycle arrestin p21 and pro-apoptotic proteins Bax and caspase 3/9 are involved (Xu *et al*., 2016). Similarly, suppression of VEGF expression leading to slower tumor progression was documented (Xu *et al*., 2016). Readers keen to see a recent summary of our knowledge of glucan's role in cancer inhibition should read some recent excellent reviews (Vannucci *et al*., 2013; Sima *et al*., 2015).

Decades of intensive research revealed significant biological effects of β-glucans. First, the attention was focused on immunostimulation, from stimulation of hematopoiesis (Patchen *et al*., 1988) to potentiation of anti-infectious immunity (Hadden, 1987; Spruijt *et al*.,

2010) and finally suppression of cancer development (Nakao *et al*., 1983; Hong *et al*., 2003). Besides these well-established effects, *β*-glucan was also found to improve HDLC and diminish LDLC and non-HDL cholesterol levels in overweight individuals with mild hypercholesterolemia (Reyna-Villasmil *et al*., 2007) to potentiate the methotrexate treatment of adjuvant arthritis (Rovensky *et al*., 2011) and to improve wound healing. These claims have already resulted in a commercially available *β*-glucan-collagen matrix that combines *β*-glucan with collagen and has proven to have excellent results in the successful treatment of burns in children (Delatte *et al*., 2001).

More and more attention has been focused on the role of *β*-glucan in inflammation. Some additional and lesser known effects of *β*-glucan include the attenuation and even the prevention of experimental colitis. Several experimental studies showed that orally-administered *β*-glucan caused significant inhibition of inflammation, production of pro-inflammatory cytokine interleukin 1 beta and colon shortening. Some studies have shown that *β*-glucan plays an important role as an immunomodulator in the treatment of ulcerative colitis (Nosalova *et al*., 2001). We cannot overlook the effects of glucan on stress-besides direct effects on stress reduction (Vetvicka and Vetvickova, 2014), clinical trials also found improvements of physical conditions in children under stress (Richter *et al*., 2015).

Lately, *β*-glucans are finding their role not "only" in immunostimulation and overall health improvement, but also in nutrition. A study on fish showed that using *β*-glucan as a probiotic supporting the activity of *Lactobacillus* significantly lowered mortality from *Aeromonas* challenges (Ngamkala *et al*., 2010). However, more has been done on the prebiotic front. An interesting study showed that the addition of *β*-glucan and starch during cold storage strongly increased survival of bifidobacteria strains in yogurt, most probably due to the protective effects on bifidobacteria stress by low temperature (Rosburg *et al*., 2010). Another *β*-glucan with high prebiotic properties was isolated from *Lactobacillus plantarum* (Das *et al*., 2014). Similar effects were obtained when shrimp was used as an experimental model.

The situation in calves is similar: Feeding with tylosin and *β*-glucan as prebiotics has positive effects on selected humoral immunological parameters, including the total protein and gammaglobulin concentration (Szymanska-Czerwinska and Bednarek, 2011). Prebiotic potential of *β*-glucan was also tested on humans. A randomized, double-blind, placebo-controlled clinical study was aimed to evaluate the *in vivo* prebiotic potential of *β*-glucan. Fifty-two healthy volunteers were assigned to consume a *β*-glucan or placebo daily for 30 days. In volunteers over 50 years of age, *β*-glucan induced a strong bifidogenic affect and increase of bifidobacteria. The authors of this study concluded that the daily intake of a *β*-glucan was not only well-tolerated, but demonstrated strong bifidogenic properties in older, healthy volunteers consuming their usual food (Mitsou *et al*., 2010).

Conclusion

It is clear that it took polysaccharides a long time to reach their true potential. Even if it is true that after centuries of sometimes intensive research, our knowledge of their biological effects is far from optimal, there are no doubts about their importance. However, the process of accepting natural molecules is a long process, it took over 70 years to accept that vitamin C cures scurvy. It may take repetitious studies and years or even decades before individual polysaccharides will be credited for their role in health improvements.

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Author's Contributions

All authors equally contributed to this study.

Conflict of Interest

Authors declare no conflict of interest.

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