CHAPTER 6 IMMUNOMODULATORY ACTIVITIES OF MUSHROOM GLUCANS AND POLYSACCHARIDE-PROTEIN COMPLEXES IN ANIMALS AND HUMANS

Synopsis:

This chapter focuses on the ability of extracts from medicinal mushrooms to stimulate or modulate the host immune responses. Numerous bioactive polysaccharides or polysaccharide-protein complexes from medicinal mushrooms are described that appear to enhance innate and cell-mediated immune responses, and exhibit antitumour activities in animals and humans. Stimulation of the host immune defence systems by bioactive polymers from medicinal mushrooms has significant effects on the maturation, differentiation and proliferation of many kinds of immune cells in the host. Many of these mushroom polymers were reported previously to have immunotherapeutic properties by facilitating growth inhibition and destruction of tumour cells. Recent research has also shown that some of these mushroom-derived polymers may possess direct cytotoxic effects on cancer cells. Whilst the mechanism of their antitumour actions is still not completely understood, stimulation and modulation of key host immune responses by these mushroom polymers appears central. Although somewhat controversial, recent evidence suggests that mushroom polymers (β-glucans) may trigger the stimulation of many kinds of immune cells in animals and humans by binding to a specific cellular receptor known as complement receptor type 3 or CR3.

Overview of the human immune system

A general overview of the immune system is provided in Appendix I. This has been provided so that an appreciation can be gained of how biological molecules from certain medicinal mushrooms may modulate the immune responses. Readers who are not familiar with the workings and complexities of innate (non-specific) and acquired (specific) immunity are advised to consult Appendix I before reading this chapter.

Medicinal mushrooms and the human immune response

While the historical and traditional usage of the medicinal mushrooms, especially in the Far East, is almost limitless (Hobbs, 1995), this chapter will largely focus on presenting well-investigated findings from the last three decades. Indeed, the oldest written record of mushrooms as medicinals is in an Indian medical treatise from 3000 BC (Kaul, 1997). Of significant relevance and importance is the ability of particular mushroom-derived compounds to modulate the human immune response and to inhibit certain tumour growths (Wasser and Weis, 1999a,1999b).

Medicinal mushroom research has focused on discovering compounds that can modulate positively or negatively the biologic response of immune cells. Those compounds which appear to stimulate the human immune response are being sought for the treatment of cancer, immunodeficiency diseases, or for generalised immunosuppression following drug treatment; for combinational therapy with antibiotics; and as adjuncts for vaccines (Jong *et al*., 1991). Those compounds that suppress immune reactions are potentially useful in the remedy of autoimmune (an abnormal immune response against self-antigens) or certain gastro-intestinal tract diseases (e.g. Crohns) (Badger, 1983). Several classes of compounds, such as proteins, peptides, lipopolysaccharides, glycoproteins, and lipid derivatives, have all been classified as molecules that have potent effects on the immune system (Tzianabos, 2000). Whilst polysaccharides are generally considered to be classic T-lymphocyte-dependent antigens that do not elicit cell-mediated immune responses (host defences that are mediated by antigen-specific T lymphocyte cells and various non-specific cells of the immune system), certain polymers have recently been shown to act as potent immunomodulating agents (Tzianabos, 2000). Compounds that are capable of interacting with the immune system to upregulate or downregulate specific aspects of the host response, can be classified as immunomodulators. Whether immunomodulators enhance or suppress immune

responses can depend on a number of factors such as dosage, route of administration, and timing and frequency of administration (Tzianabos, 2000). The type of activity these compounds exhibit can also depend upon their mechanism of action or the site of activity.

Immunomodulating polysaccharides derived from a variety of diverse microbial genera include *Streptococcus* spp. (hyaluronic acid), *Bacteriodes fragilis* (Polysaccharide A), *Candida albicans* (Mannan) and *Saccharomyces cerivisiae*, have shown significant promise in the treatment of infectious diseases (Garner *et al*., 1990; Shapiro *et al*., 1986; Muller *et al*., 1997; Schrager *et al*., 1998). Antitumour effects were another promising biopharmacological activity of polysaccharides from these sources. The earliest bacterial-derived polysaccharide reported to have antitumour activity was attributed to *Serratia marcescens* and became known as Shear's polysaccharide (cited in Ooi and Liu, 2000). This polysaccharide could cause extensive cytotoxic damage to Sarcoma 37 tumours, but as it had serious side-effects, clinical trials have not been performed. Although many other polysaccharides from bacteria such as *Escherichia coli*, *Streptococcus pyogenes* (OK-432), *Proteus vulgaris*, *Acetobacter xylinum* and *Salmonella typhimurium* have also been reported to exhibit cytotoxicity against solid tumours (Whistler *et al.,* 1976); however most of these bacterial polysaccharides belong to endotoxic lipopolysaccharides.

One of the most significant factors of many of the derived bioactive polymers from medicinal mushrooms is their role as immunomodulators. Whilst information will be presented in this report that demonstrates the ability of certain medicinal mushroom (MM) extracts to modulate key components of the immune system, it is appropriate at this point to mention a number of important immune responses that are stimulated by some of these bioactive polymers. As described in Appendix I, the

immune system plays an important role in the body's defence against infections and tumour formation. Moreover, the body's defence against viral attack and against spontaneously arising malignant tumour cells comprises a dynamic orchestrated interplay of innate and acquired immune responses. Innate immunity (having macrophages, neutrophils, NK and dendritic cells as gatekeepers), is regulated by chemical-messengers or cytokines and by activation of inflammatory and acute phase responses (Chihara, 1992). The mononuclear phagocyte system (e.g., macrophages and monocytes), dendritic cells and certain lymphocytes (e.g., natural killer cells) serve a number of important roles including the recognition and destruction of abnormal cells.

Stimulated macrophages and Natural Killer (NK) cells produce cytokines such as interferons, interleukins and others that are targeted towards destroying cancer cells. These are regarded as the first line in the host defence system, and may themselves successfully eliminate infected or transformed cells prior to the establishment of fully-fledged humoral and CMI responses (Borchers *et al*., 1999). As described in Appendix 1, specific immunity to abnormal cells or tissues includes humoral (e.g., generates antibodies) and cell-mediated immunity (also promotes inflammatory responses and ultimately kills infected or abnormal cells). As a fully functional immune response is critical to the recognition and elimination of tumour cells, the identification of mushroom derived compound(s) that are capable of stimulating components of innate or acquired immunities may be of potential benefit for cancer treatment.

Thus these immunological activities play a governing role in host recognition, targeting and destroying unwanted tumour-potentiating viruses and abnormal or cancerous cells. Induction and expression of cellular immunity in host resistance to cancer and persistant microbial infections is contingent upon a myriad of complex

interactions between antigen, macrophages, and lymphocytes (Borchers *et al*., 1999). It is through the orchestrated interplay of many of these innate and specific immune responses that abnormal cells are targeted and destroyed. For example, the key immune mechanisms that are involved in Lentinan (a polysaccharide from *L. edodes*) mediated destruction of cancer cells are illustrated in Figure 1 (Chihara, 1992). Tumours may develop when transformed cells escape immunological host defence mechanisms (Ooi and Liu. 1999, 2000). Indeed, the increased incidence of spontaneous tumours in immunosuppressed individuals (as well as those congenital or acquired immunodeficiencies), indicates that the immune system can provide a significant mechanism for host resistance against cancer and infectious diseases (Jong *et al*. 1991).

The ability of bioactive polysaccharides and polysaccharide-bound proteins to modulate so many important immune cells may due to the structural diversity and variability of these macromolecules. Unlike proteins and nucleic acids, polysaccharides contain repetitive structural features which are polymers of monosaccharide residues joined to each other by glycosidic linkages (Ooi and Liu, 2000). Among these macromolecules, polysaccharides offer the highest capacity for carrying biological information because they have the greatest potential for structural variability. For example, the number of possible permutations for four different sugar monomers can be up to 35,560 unique tetrasaccharides, whereas four amino acids can form only 24 different permutations (cited in Ooi and Liu, 2000). Therefore, this enormous potential variability in polysaccharide structure gives the necessary flexibility for the precise regulatory mechanisms of various cell-cell interactions in Higher organisms.

Immuno-modulating effects of *Lentinus edodes* mycelium (LEM) extract and Lentinan

Of all the mushroom immune modulators investigated, bioactive polymers from *Lentinus edodes* has been studied extensively for interesting biological effects. Moreover, *L. edodes* is the source of two preparations with well-studied pharmacological effects – *Lentinus edodes* mycelium (LEM) extract and lentinan (Hobbs, 2000). Lentinan (a cell wall constituent extracted from fruiting bodies or mycelium) is a highly purified, high molecular weight polysaccharide in a triple helix structure containing only glucose molecules with mostly β-(1→3)-Glc linkages in the regularly branched backbone, and β-(1→6)-Glc side chains (Aoki, 1984, Hobbs, 2000). The configuration of the glucose molecules in a helix structure is thought to be important for the biological activity (Hamuro et al., 1971). Lentinan is protein-free as it is completely devoid of any nitrogen, phosphorous, sulphur or any other atoms of carbon, oxygen, and hydrogen (Hobbs, 2000). Lentinan is water-soluble, heat stable, acid stable and alkali labile (Hobbs, 2000). LEM is a preparation of the water-soluble material from powdered mycelia extract of *L. edodes* harvested before the mushroom fruiting bodies develop. The major active constituent of LEM is reported to be a heteroglycan protein conjugate, that is, a protein-bound polysaccharide. It contains about 24.6% protein and 44% sugars, in addition to nucleic acid derivatives and vitamins (Breene, 1990, Iizuka, 1997). Other active polysaccharides and protein-polysaccharide complexes and water-soluble lignins were isolated from LEM (Tabata et al., 1992).

Lentinan does not attack cancer cells directly, but produces its antitumour effect by activating different immune responses in the host. Recent research has shown that LEM and Lentinan are true immuno-potentiators, as administration of these bioactive polymers had a clearly augmenting effect on the proliferation of

peripheral mononuclear cells (PMNCs) from healthy donors, (Aoki, 1984, Hobbs, 2000). Indeed, Lentinan and LEM appear to act as a host defense potentiator that is able to restore or augment the responsiveness of host cells to lymphocytokines, hormones, and other biologically active substances. Evidence suggests that this immune-potentiation occurs by stimulating the maturation, differentiation or proliferation of cells involved in host defense mechanisms. Thus, Lentinan has been shown to increase host resistance against various kinds of cancer and has the potential to restore the immune function of affected individuals (Chihara, *et al*., 1989, 1992). Many of these immune pathways stimulated by Lentinan are illustrated in Figure 1.

Lentinan has displayed various kinds of immune activities in both animals and in humans (Table 1). Until recently, the interactions of Lentinan with many kinds of immune cells were not known. An insight into receptor-binding in immune cells by β-glucans from fungi was provided by Ross et al. (1999). These authors showed that β-glucans from yeast bind to iC3b-receptors (CR3, CD11b/CD18) of phagocytic cells and natural killer (NK) cells, stimulating phagocytosis and/or cytotoxic degranulation. Further information of receptor-base binding and affinity for many kinds of immune cells is provided later in this chapter. Thus, research has shown previously that Lentinan stimulates various kinds of immune cells including macrophages, NK-cells and lymphocytes (T and B cells).

The anti-tumour activity has been shown to be abolished in neonatally thymectomised mice and was decreased by the administration of antilymphocyte serum. Both practices reduce or eliminate T lymphocyte production that is central to cell-mediated immunity. This supports the concept that Lentinan requires

Table *1* ***Immune effects of Lentinan in vitro and in vivo in animals and humans*** *(Hobbs, 2000)*

Activity	Experimental Animal System		Human System	
	in vitro	**in vivo**	**in vitro**	**in vivo**
Humoral factors				
Inhibition of immunosuppressive factors	–	++	–	++
Immunopotentiative factors, increased production	–	++	–	–
C3-splitting activity	–	+	–	–
Antibody production	–	+	–	+
Opsonin production	–	–	–	+
Colony-stimulating factor production	+	–	–	–
Production of lymphocyte-activating factor (interleukin-1)	+	+	+	+
Inhibition of prostaglandin release	–	+	–	–
Interferon production	–(?)	+	–±	–
Tumor necrosis factor production increased	–	+	–	–
Complement C3 production	–	–	–	+
Cellular factors				
Polymorphonuclear leukocyte activation	–	+	+	+
Peritoneal macrophage activation	–	+	–	–
Natural killer cell activation	+	+	±~+	++
Activation of helper T cells	–	+	+	++
Activation of killer T cells	+	+	+	–
Inhibit suppressor T-cell activity	–	+	–	–
Activation of cytotoxic macrophages	–	+	–	+
Delayed-type hypersensitivity reaction	+	+~++	–	–
Mitogenicity	–	–	±~+	++

immunocompetent T-cell compartments (Maeda *et al*., 1971; Maeda and Chihara, 1973). The effect of Lentinan was also inhibited by anti-macrophage agents such as carrageenan. Unlike other well-known immuno-stimulants, Lentinan is in a unique class of DT-cell-oriented assistants, in which macrophages play some part. For example, Lentinan can activate NK-cells *in vitro* in the same concentrations that are achieved in the blood plasma of patients treated clinically with Lentinan. NK cell activity is involved in tumour suppression and while these cells do not stimulate certain T-killer cell activity, or do so only under certain conditions, they are strong T-helper cell stimulants both *in vitro* and *in vivo*. Lentinan can inhibit prostaglandin synthesis, which can slow T-cell differentiation in animals and humans, as well as inhibiting suppressor T-cell activity *in vivo* (Aoki, 1984), and in addition, increase the

ratio of activated T cells and cytotoxic T cells in the spleen when administered to gastric cancer patients with chemotherapy (Hobbs, 2000).

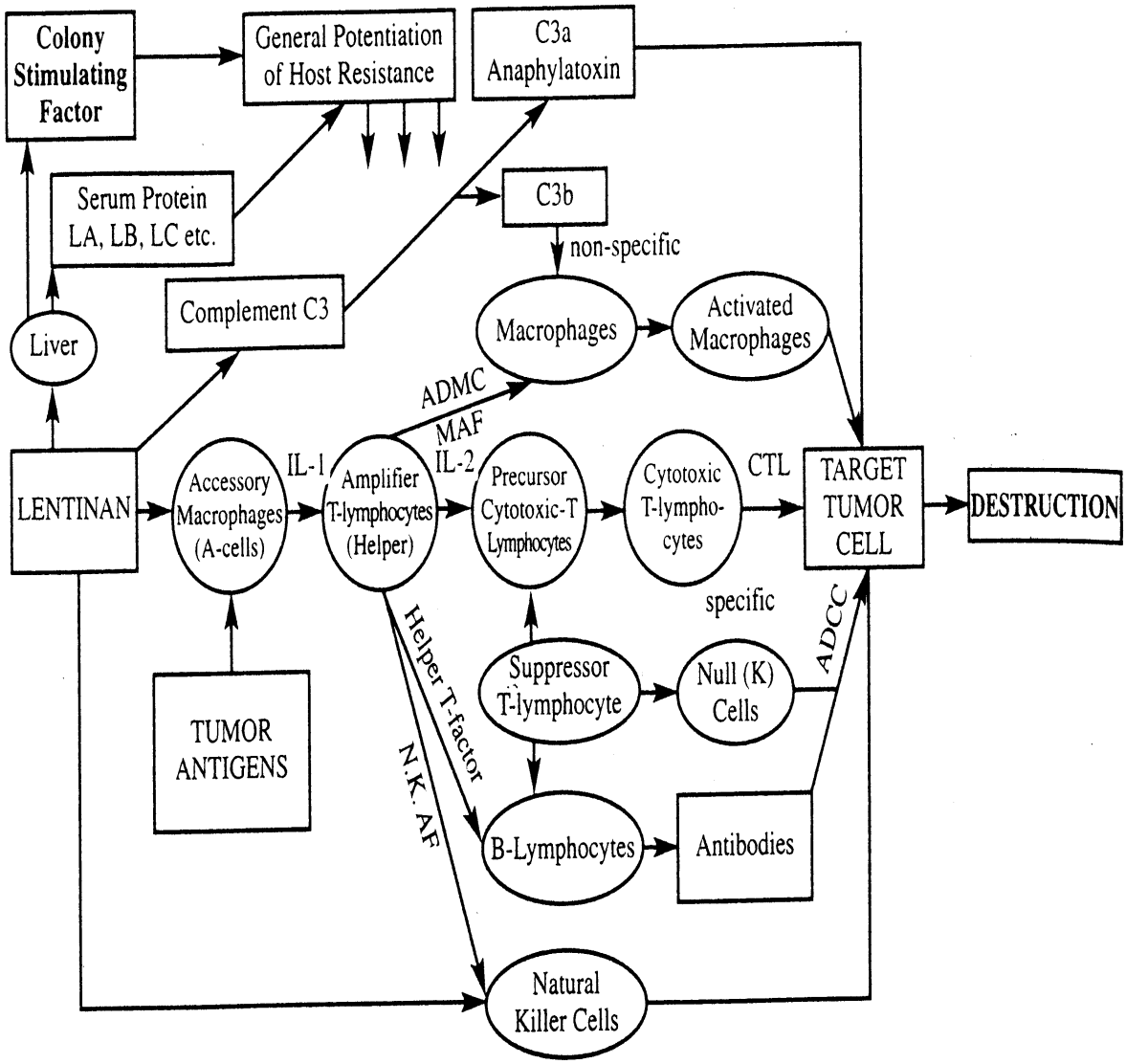
Using the blood of healthy donors and cancer patients, Lentinan has been shown to stimulate peripheral blood lymphocytes *in vitro* to increase interleukin-2-mediated LAK-cell (lymphokine-activated killer cell) and NK cell activity at levels achievable *in vivo* by administration of clinical doses of Lentinan. Lentinan has also been shown to inhibit suppressor T cells activity *in vivo* and to increase the ratio of activated T cells and cytotoxic T cells in the spleen when administered to gastric cancer patients undergoing chemotherapy.

Many interesting biological activities of Lentinan have been reported (Figure 2), including:

- An increase in the activation of non-specific inflammatory response such as acute phase protein production (Suga *et al*, 1986)
- Vascular dilation and haemorrhage-inducing factor *in vivo* (Maeda *et al*, 1991)
- Activation and generation of helper and cytotoxic T cells (Chihara *et al* 1992)

Augmentation of immune mediators like interleukin-1 (IL-1) (Fruehauf *et al*., 1982), IL-3 (Izawa *et al*., 1984), IL-6 (Maeda *et al*., 1992), colony stimulating factor(s) (Izawa *et al*., 1984), and others. These serum factors are mainly produced by macrophages or T-lymphocytes and act on lymphocytes, hepatocytes, vacular endothelial cells, and other cells. Lentinan has been shown previously to increase the capacity of peripheral blood mononuclear cells of patients with gastric cancer,

Figure 1. ***Host immune responses involved in Lentinan-mediated destruction of cancer cells*** **(**Chihara *et al*., 1992)



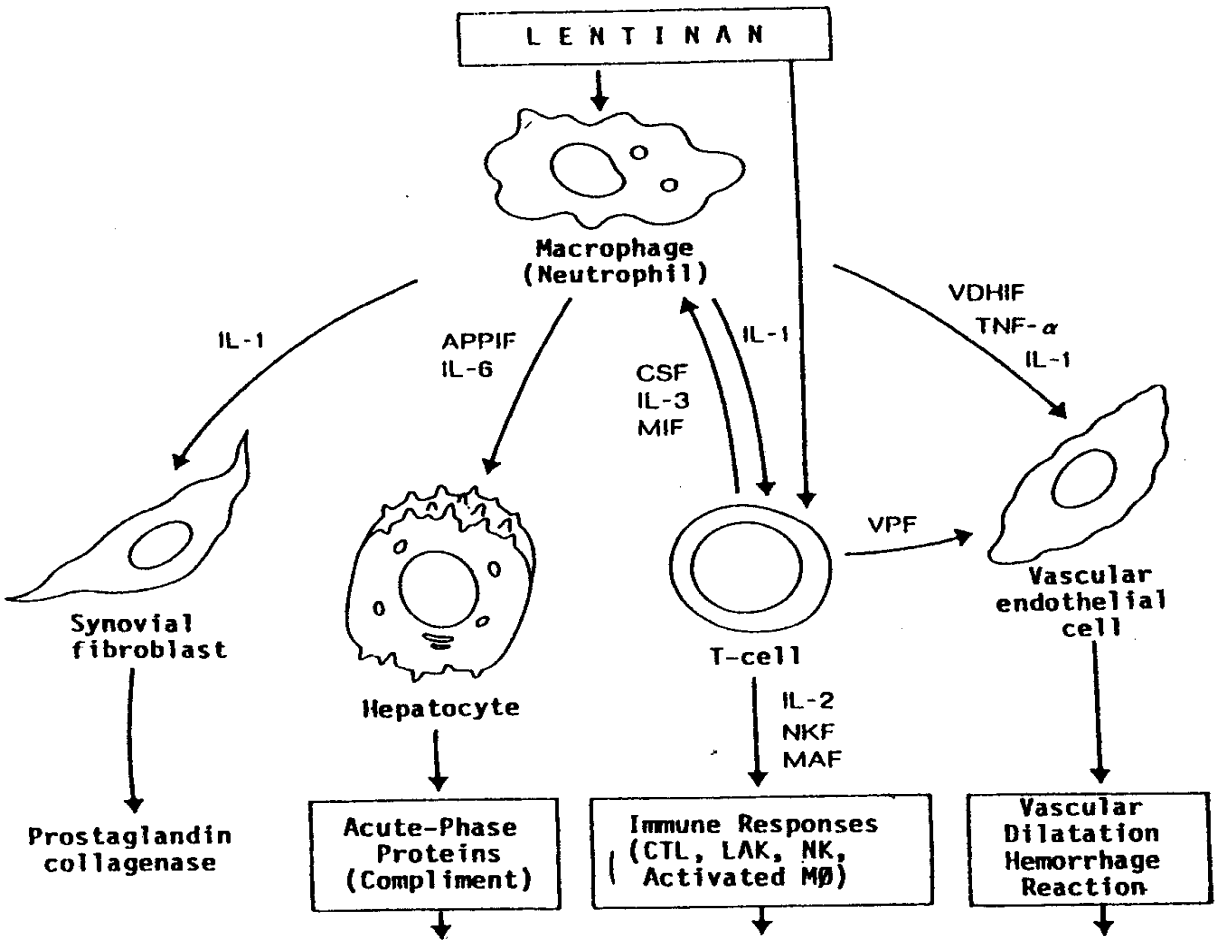
resulting in the production of IL-1α, IL-1β and TNF-a (Chihara *et al*., 1992). In its role as a host defence potentiator, Lentinan triggers the increased production of colony stimulating factors (CSFs) and IL-3, which correlates with the IL-1-producing activities of macrophages (Izawa *et al*., 1984; Chihara *et al*., 1989). Increased production of IL-1 results in augmented maturation capable of inducing IL-2, natural killer activating factor, and macrophage-activating factor (MAF). IL-1 also amplifies

maturation of immature effector cells to mature cells and augments responsiveness to lymphcytokines such as IL-2, MAF and others.

Lentinan's immune-activating ability may be linked with its modulation of hormonal factors, which are known to play a role in tumour growth. The anti-tumour activity of Lentinan is strongly reduced by administration of thyroxine or hydrocortisone. Lentinan can also restore a tumour-specific antigen-directed delayed-type hypersensitivity (DTH) response. Indeed, Lentinan is not formally included among the non-specific immunostimulants (RES stimulants), but it augments the induction of antigen-specific cytotoxic T-lymphocytes, macrophages, and other non-specific immune responses. An overview of the host immune responses involved in Lentinan-mediated recognition and destruction of cancer cells is presented in Figure 1. Interestingly, accumulating evidence suggests that Lentinan-stimulation of dendritic cells (antigen-presenting cells that are found in lymph nodes, spleen and thymus; follicular and interdigitating dendritic cells, skin: Langerhans cells, and other tissues; interstitial dendritic cells) has an important impact on immunomodulation and anti-tumour activity. Moreover, dendritic cell tumour-infiltration in association with killer cytotoxic T cell stimulation and activation have been shown to have a governing role in tumour attack and elimination (Chihara, 1997).

Bohn and BeMiller (1995) reported that the likely mode of immunopotentiation by this (1-3)-β-D-glucan involves activation of cytotoxic macrophages, helper T cells and NK cells, and the promotion of T cell differentiation. Macrophages are one of the many critical components in the immune system, co-operation between which is necessary for tumour rejection. Bohn and BeMiller (1995) also reported that macrophages have a highly selective cytotoxicity towards cancer cells *in vitro*; and

Figure 2. Early phase of the mechanism of action of Lentinan and possible pathway for inflammatory and immune reactions. IL-1, IL-2, IL-3, and IL-6: interleukin-1, -2, -3, and –6. APPIF: acute phase protein-inducing factor VDHIF: vascular dilation and hemorrhage-inducing factor; CSF: colony-stimulating factor; MIF: migration inhibition factor;' TNF: tumor necrosis factor; VPF: vascular permeability factor; NKF: natural killer cell-activating factor; MAF: macrophage-activating factor; CTL: cytotoxic T lymphocyte; LAK: lymphokine-activated killer cell (Maeda *et al*. 94)



there is evidence that they may also destroy malignant cells *in vivo*. T cell competence appears necessary for selection of macrophage resistance, which suggests that these two cell types interact in the intact host in response to a tumour challenge.

Thus, Lentinan has been shown to restore or augment the ability of host cells to respond to lymphocytokines or other intrinsic bioactive factors and protect patients

from infectious disease or cancer metastases (Chihara, 1971). Lentinan can also improve the physiological constitution of host defence mechanisms by restoring homeostasis and enhancing intrinsic resistance to disease. Homeostasis is a term given to cellular processes, by which both negative and positive control are exerted over the values of a variable or set of variables, and without which control the system would fail to function. To summarise, Lentinan may restore and augment immunological responsiveness of host cells, but it has no direct cytotoxicity against tumours.

Immunomodulating effects of Ganoderma lucidum

Ganoderma lucidum has been used extensively as "mushrooms of immortality" in China and other Asian countries for 2000 years (Shiao *et al*, 1994). Several major substances with potent immuno-modulating action have been isolated from this mushroom, including polysaccharides (in particular β-D-glucan), proteins (e.g., Ling Zhi-8) and triterperoids (Jong and Birmingham, 1992; Gao and Zhou, 2001). Other components such as steroids and organic gemanium also play an important role in the immuno-modulating activity of *G. lucidum*. The major immuno-modulating effects of these active substances derived from *G. lucidum* include mitogenicity and activation of immune effector cells such as macrophages, NK and T cells (Gao and Zhou, 2001). Stimulation of these immune effector cells results in the production of cytokines such as interferon (INF), interleukins (IL) and tumour necrosis factor (TNF)-α.

More than 100 types of polysaccharides have been isolated from *G. lucidum*. β-D-glucans (i.e., polysaccharides producing D-glucose by acid hydrolysis) have been shown to be biologically active (Mizuno *et al*., 81; Mizuno *et al*., 1982; Gao, 2000). Modification of D-glucosyl groups of side chains of β-D-glucans enhanced

anti-tumour activity. There is evidence that the β-D-glucans induce biological response by binding to membrane complement receptor type three (CR3, $\alpha_M\beta 2$ integrin, or CD11b/CD18) on immune effector cells such as macrophages (Battle *et al*., 1998; Mueller *et al*., 2000). The β-glucan binding site of CR3 has been mapped to a region of CD11b located at the C-terminus of the I-domain. The ligand-receptor complex can be internalised, and the intercellular events that occur after glucan-receptor binding have been determined (Muller *et al*., 1996). Preliminary evidence shows that the NF-κB is activated (Battle *et al*., 1998). β-D-glucan can also help override the normal resistance of iC3b-opsonized tumour cells to the cytotoxic activation of phagocyte and NK cell CR3, allowing this important effector mechanism of the complement system to function against tumour cells (Ross *et al*, 1999; Xia *et al*., 1999).

More than 100 triterpenoids have been isolated from the fruiting body and mycelia of *G. lucidum*, which include highly oxidised lanostane-type triterpenoids (e.g., ganoderic acid, lucidenic acid, ganodermic acids, ganoderenic acids, lucidone, ganoderal, and ganoderols) (Kim and Kim, 1999; Wasser and Weis, 1999a). Some of these triterpenoids have shown immuno-modulating activity (Kim and Kim, 1999). Immunomodulating proteins, such as Ling Zhi (LZ)-8, has been isolated from *G. lucidum*. (Tanaka *et al*., 1989). The major biological activities of LZ-8 resemble lectins, with mitogenic activity towards mouse spleen cells and human peripheral lymphocytes and agglutination of sheep red blood cells *in vitro*. Recently, immuno-modulatory protein (Fip-gts) has been purified from *G. tsugae* (Lin *et al*., 1997).

Extracts from *G. lucidum* containing poylsaccharides and LZ-8 have shown mitogenic effects on human peripheral blood mononuclear cells (PBMC) (King *et al*., 1989). Both *in vitro* and *in vivo* studies in mice have shown that water-soluble

extracts from *G. lucidum* can stimulate the production of interleukin (IL)-2 by splenocytes in the presence of hydrocortisone (Zhang *et al*., 1993).

Crude, water-soluble extracts from *G. lucidum* have been previously shown to be potent activators of human T lymphocytes, where they induce the production of cytokines such as IL-1β, INF-γ, TNF-α, IL-2, IL-6 and IL-10 (Wang *et al*., 1997; Mao *et al*., 1999). A polysaccharide fraction from *G. lucidum* (GLB) was shown to promote the production of IL-2 in a dose-dependent manner. GLB augmented the toxicity of cytotoxic T lymphocytes by as much as 100% when administered at a concentration of 200 μg/ml (Lei and Lin, 1992). LZ-8 also mediates T cell activation via cytokine regulation. Haak-Frendscho *et al*. (1993) showed that stimulation of PBMC by LZ-8 results in the production of IL-2 and a commensurate up-regulation of IL-2 receptor expression. LZ-8 also induces aggregate formation in PBMC, which is correlated to a marked rise in ICAM (intercellular adhesion molecule)-1 expression and to an increased production of INF-γ, TNF-α and IL-1β. The addition of neutralising monoclonal antibodies to IL-2 receptor and TNF-α blocks cellular aggregate formation and proliferation, and ICAM-1 expression.

A water-extracted polysaccharide fraction from *G. lucidum* enhanced the cytotoxicity of splenic NK cells in tumour-bearing mice (Won *et al*., 1989, Lee *et al*., 1995b). Murine and human macrophages are also activated by polysaccharides from *G. lucidum* (Lee *et al*., 1995b). The macrophage responses (such as the release of cytokines, nitric oxide and other mediators) are associated with anti-tumour and anti-inflammatory effects. CR3 receptors on human macrophages bind β-D-glucans and become internalised. This initiates a cascade of events including the production of IL-1β, IL-6, INF-γ and TNF-α which cause anti-proliferation and the induction of apoptosis in HL-60 and U937 leukemic cells (Lee *et al*., 1995b; Wang *et al*., 1997). Antibody neutralisation studies have shown that INF-γ and TNF-α

released from macrophages act synergistically to inhibit the growth of leukemic cells (Li *et al*., 2000).

A β-D-glucan (Ganoderan) and a protein-polysaccharide fraction (GLB) from *G. lucidum* are potent stimulators of mice and chicken macrophages. Ganoderan and GLB have been shown to increase the expression of MHC class II molecules on these antigen-presenting macrophages (Oh *et al*, 1998). There is also evidence to suggest that extracts from *G. lucidum* can influence humoral or B cell immunity. *In vivo* studies have shown that repeat administration of LZ-8 at 8-12 mg/kg reduces antibody production in mice (Kino *et al*, 1991). Lee *et al*. (1990) showed that an alkali extract from *G. lucidum* activated both the classical and alternative pathways of the complement system. This extract also activated the reticuloendothelial system and increased haemolytic plaque forming cells in the spleen of mice. A clinical study in aged patients with insomnia and palpitations recently showed that the consumption of *G. lucidum* extract for up to 6 weeks increased their serum C3 levels (Yang and Pai, 2000).

Various substances from *G. lucidum* (e.g., polysaccharides, triterpenoids, and proteins) have been shown to have marked immuno-modulating effects such as augmenting the activity of effector T cells, NK cells and macrophages. To-date, no pure β-glucan product derived from this particular mushroom has been commercially available. However, β-glucan appears to be one of the first biological response modifiers for which the cellular mechanism of action has been initially defined at the specific receptor level. As the cytotoxic host defence function of β-glucan is specific for target cells bearing iC3b, its' action in promoting host defense relies on the specificity of the antibody.

Immunomodulating effects of polysaccharides PSP and PSK from **Trametes versicolor**

Protein bound polysaccharides PSK (Krestin) and PSP have been isolated from the mushroom *Trametes versicolor*. These compounds are chemically relatively similar and have a molecular mass of about 100 kDa. The polysaccharide component is made up of monosaccharides with α-(1-4) and β-(1-3) glucosidic linkages. PSK and PSP differ mainly in the presence of fucose in PSK and rhamnose and arabinose in PSP. Both PSK and PSP are potent immunostimulators with specific activity for T-cells and for antigen-presenting cells such as monocytes and macrophages. The biologic activity is characterized by their ability to increase white blood cell counts, IFN-γ and IL-2 production and delayed type hypersensitivity reactions (Tzianabos, 2000). Numerous reports have documented the ability of PSK and PSP to activate cellular and humoral components of the host immune system. In addition, these polysaccharides have been shown to inhibit the growth of tumour cell lines and to have in vivo anti-tumour activity (Tzianabos, 2000). As there is considerable information on the immunomodulating activities of both PSP and PSK, they will be discussed separately.

The effect of PSP on the phagocytic functions has been tested in normal ICR mice. It was determined that the carbon clearance rate of the groups given oral (p.o.) doses of 0.5-1.5 g PSP/kg or intraperitoneal (i.p.) injections of 100, 200, 400 mg/Kg was similar to that of groups treated with acanthopanax (300 mg/kg). Regardless of the route of administration PSP increased the carbon clearance rate in mice, suggesting that PSP can appreciably increase the phagocytic function of normal animals (Jong and Yang, 1999; Yang *et al*., 1993). *In vitro* experiments with spleen T-lymphocytes cultured in solutions containing various concentrations of PSP showed that, when compared to the physiological saline control group,

concentrations in excess of 100 μg/ml produced an increase by a factor of 1.5 to 4 times in T-lymphocytes. It was also determined that PSP can appreciably increase the secretion of IL-2 in mice (Yang *et al*., 1993). Human white blood cells (WBCs) were cultivated in solutions containing different concentrations of PSP. Using vesicular stomatis virus as the challenge virus, the PSP induced interferon in human WBCs. γ-interferon levels in the PSP group were twice those of the control group, while α-interferon levels were two to four times higher. PSP can promote the expression of the IL-6 gene of peripheral blood lymphocytes (PBL) in humans and hence induce the production of interleukin 6 (IL-6) (Yang *et al*., 1993; Yu *et al*., 1996).

It has been recently reported that PSP in different concentrations promoted the proliferation of T-lymphocytes both in human peripheral blood and mouse splenocytes (Li *et al*., 1999). It appeared that human T-cells were more sensitive to PSP than that of mouse lymphocytes, a finding that was corroborated by Ling and Wang (1996). PSP augmented T-helper cell (CD_4^+) activation, and also increased the ratio of CD4+/T suppressor (CD8+) production. Li (1999) also showed that while the chemotherapeutic drug cyclophosphamide (CPA) inhibited the delayed type hypersensitivity (DTH) response in mice, administration of PSP and CPA together restored the DTH response in mice to normal levels, suggesting that PSP negates that inhibitory action of CPA in treated mice. CPA was first reported in 1958 and has become the leading drug of the alkylating class in the clinical treatment of cancer particularly for lymphomas, leukemias and a variety of solid tumours (Struck *et al*., 1995; Yule *et al*., 1996). Alkylating agents including CPA are cytotoxic, rapidly kill dividing neoplastic and normal cells and have drastic effects on cells on the immune system. Both humoral and cellular immune function may be inhibited to a varying degree by CPA (Awward *et al*., 1995). Indeed, it has been documented that CPA

reduces the ability of B lymphocytes to mount an appropriate antibody response *in vivo* and *in vitro*. CPA also reduces circulating levels of leukocytes and induces immunological function disturbances such as impaired phagocytic capability, release of lytic enzymes and inhibition of NK cell activity (Turk and Poulter, 1972; Dumont, 1974; Eremin; 1992, Qian *et al*., 1999).

PSP was shown to enhance B cell function (humoral immunity) in normal and tumour bearing mice that had been challenged with foreign antigen (i.e., sheep red blood cells). PSP increased the levels of haemolysin (antibody) in treated mice, this response was particularly pronounced in tumour bearing–mice (Zhou *et al*., 1988). PSP influenced macrophage response as it significantly increased both the clearance index and phagocytic index of mice that were intravenously challenged with charcoal particles. Enhanced production of IL-1, IL-6 and INF was also observed in activated macrophages (such as) from charcoal-challenged mice. While the use of PSP had no significant effect on NK activity in radio-labelled K562 tumour cells, it did increase NK activity in tumour cells that had been treated with cyclophosphamide. Treatment of K562 tumour cells with cyclophosphamide alone was shown to inhibit NK activity. This may explain in part, why PSP has been shown to improve the immune condition of patients receiving chemotherapy. Indeed, clinical studies showed that PSP treatment increased the activity of NK cells by an average of 64.5% in 138 cancer patients (Yang and Li, 1993).

Recent studies showed that PSP stimulates lymphokine-activated-killer (LAK) cell proliferation, and reduces the concentration of IL-2 needed to produce a cytotoxic response (Jian *et al*, 1999). Qian *et al*. (1999) also showed that PSP (2g/kg/day) possessed immunopotentiating activities, being effective in restoring cyclophosphamide (CPA) induced immunosuppression such as depressed lymphocyte proliferation, NK cell function, production of white blood cells and the

growth of spleen and thymus in rats. In addition, PSP increased both IgG and IL-2 production where CPA had significant inhibitory effects. PSP effectively stimulated the generation of INF-α reaching levels of 800 – 1000 IU/ml when the concentration of PSP was 100 μg/ml, and improved yields of INF-γ were reported (Yang *et al*., 1999). PSP in concentrations of 50-100 μg/ml promoted the proliferation of phytohaemagglutinin (PHA) - activated human peripheral blood lymphocytes (Liang *et al*., 1999). These researchers observed a greater increase in the CD_4^+ cell group levels compared with CD_8^+ cells, thereby raising CD_4^+/CD_8^+ ratio.

Macrophages play a pivotal role in non-specific immunity and can be activated by invading microorganisms, lymphokines, endotoxin and various cell mediators and regulators. An increase in the production of reactive nitrogen intermediates, reactive oxygen intermediates (superoxide anions) and TNF was reported by Liu *et al*. (1999) in peritoneal macrophages collected from C57 mice that had received PSP in drinking water for 2 weeks. Northern blot analysis of DNA also demonstrated that PSP activated the transcription of the tumour necrosis factor gene in these cells, indicating the PSP immunomodulatory effect on defensive cells. Liang *et al*. (1999) recently addressed the question as to whether polysaccharide-bound proteins (e.g., PSP) act by exerting cytotoxicity on tumour cells or by regulating the immune responses of effector cells such as macrophages. This showed that PSP at concentrations of 2.5-10 μg/ml did not exert any cytotoxicity on cultured mouse peritoneal macrophages nor on five tumour cell lines consisting of two macrophage-like cells (PU5 and P338D1), a human choriocarcinoma cell line (JAR), a mouse melanoma (B16) and sacroma (S180) cell lines. The molecular basis for the tumouricidal activity of activated macrophages is not clearly known, but their secretary products such as TNF and reactive nitrogen and oxygen intermediates may play an important role in this process (Nathan and Hibbs, 1991). Reactive

nitrogen intermediates suppress mitochondrial respiration of tumour cells, hence exhibiting their cytotoxicity against target cells (Takema *et al*., 1991). However, it is not known whether PSP directly modulates cytokine action or participates in signal transduction within macrophages.

PSK has been shown to have no substantial effect on immune responses of the host under normal conditions (Ehrke *et al*., 1983; Tsukagoshi *et al*., 1984). It can restore the immune potential to the normal level after the host was depressed by tumour burden or anticancer chemotherapeutic agents (Tsukagoshi *et al*., 1984; Dong *et al*., 1996, 1997). In ICR mice, antibody production against trinitrophenyl that had depressed the immunity in Sacroma 180-bearing mice can be restored by PSK administration. Oral administration of PSK can improve the impaired antitumour CD4+ T-cell response in gut-associated lymphoid tissue of specific-pathogen free mice (Harada *et al*., 1997). PSK enhances the cytotoxic activity of peripheral blood lymphocytes (PBLs) *in vivo* and *in vitro*. On a related issue, it may accelerate the interaction of PBL with tumour cells such as T24 human urinary bladder tumours when effector cells and target cells are exposed to PSK simultaneously.

After intra-tumoural adminstration, PSK may come into close contact with tumour cells, whereupon local inflammatory responses occur and result in the non-specific killing of these abnormal cells. Consequently, local administration of PSK is more efficient than systemic use (Mizutani and Yoshida, 1991). It has been reported that PSK induces gene expression of some cytokines such as TNF-α, IL-1, IL-8, and IL-6, *in vivo* and *in vitro* (Kato *et al*., 1995; Liu *et al*., 1996). These cytokines, produced by monocytes, macrophages, and various other cell types, mediate multiple biological effects by direct stimulation of cytotoxic T cells against tumours, enhancement of antibody production by B lymphocytes and induction of IL-2 receptor expression on T lymphocytes. The induction of TNF-α by PSK would

contribute, in part, to potent tumouricidal effects of this agent, as the administration of neutralizing antibody against TNF-α significantly attenuates the anti-tumour activity of PSK in the murine model (Kato *et al*., 1995). Interestingly, recent studies indicate that PSK exerts tumourcidal activity by inducing T cells that recognise PSK as an antigen and kill tumour cells in an antigen-specific manner (Okazaki *et al*., 1995).

The anti-tumour activity of medicinal mushrooms has been evaluated in Japan for prevention of esophageal, gastric, and lung cancers with promising results (Ng, 1998). In phase II and phase III trials in China, PSP significantly enhanced immune status in 70 to 97% of patients with cancer of the stomach, esophagus, lung ovary and cervix. In these studies, PSK and PSP increased the number of immune cells and facilitated dendritic and cytotoxic T-cell infiltration of tumours. The polysaccharides were well-tolerated and compatible with chemotherapy and radiation treatment.

Immunomodulatory activities of compounds from other medical mushrooms

Schizophyllan from *Schizophyllum commune*

In addition to the intensively researched mushrooms described previously, glucans and polysaccharide-bound protein complexes from many other medicinal mushrooms have been shown to exert immunomodulating activities *in vivo* and *in vitro*. Schizophyllan, from *Schizophyllum commune*, is relatively similar to Lentinan in composition and biological activity, and its mechanism of immunomoduation and anti-tumour action appears to be quite similar (Jong *et al*., 1991). Recently, the induction of gene expression of cytokines by schizophyllan has been studied *in vitro*

and *in vivo* (Nemoto *et al*., 1993; Okazaki *et al*., 1995). After schizophyllan is administered intraperitoneally to ICR mice, the kinetics of gene expression of cytokines is different in peritoneal exudate cells, splenocytes, and hepatocytes (Ooi and Liu, 1999). It is generally accepted that protein synthesis and gene expression of cytokines are regulated separately. Therefore, the antitumour activity of Schizophyllan is due mainly to host-mediated immune responses (Nemoto *et al*., 1993; Okazaki *et al*., 1995).

Neither Schizophyllan nor Lentinan demonstrated any anti-tumour activity against Sarcoma 180 in an *in vivo* experiment with cyclosporin A as a T cell suppressor, which suggests that an immunocompetent T cell component is necessary for developing anti-tumour activity (Kraus and Franz, 1991 and 1992). These results indicate that Schizophyllan and Lentinan are T-cell oriented immunopotentiators and, therefore, require a functional T cell component for its biological activity and that the action of (1-3)-β-D-glucans on the host's immune system might: (1) increase helper T cell production, (2) increase macrophage production, (3) bring about a non-immunological increase of the host defence mechanisms through stimulation of acute phase proteins and colony stimulating factors, which in turn effects proliferation of macrophages, PMNC, and lymphocytes and activation of the complement system (Bohn and BeMiller, 1995).

Grifolan from *Grifola frondosa*

Another (1-3)-β-glucan, Grifolan, from *Grifola frondosa* is similar to schizophyllan in primary structure (Adachi *et al*., 1990). Enhancement of mRNA levels of IL-6, IL-1 and TNF-α of macrophages by Grifolan treatment is detected *in vitro* by reverse transcription-polymerase chain reaction (RT-PCR), showing that grifolan is a novel macrophage activator that increases cytokine production (Adachi

et al., 1994; Ooi and Liu, 1999). A novel polysaccharide-bound protein (PSPC) (Mol. Wt. 15.5 KDa) has been isolated from cultured mycelia of *Tricholoma mongolicum* Imai (Wang *et al*., 1996). PSPC activated both lymphocytes and macrophages from BALB/c mice and showed no direct cytotoxic activity against fibroblasts, hepatoma cells, and choriocarcinoma cells. Similarly, an immunomodulatory and anti-tumour PSPC with a molecular weight of about 154 x 10^3 has been purified and characterised from the culture filtrates of *Tricholoma lobayense* Heim (Liu *et al*., 1995, 1996). It inhibited the growth of Sacroma 180 implanted in mice intra-peritoneally or subcutaneously, with no sign of toxicity *in vivo* (Liu *et al*., 1995). PSPC has been able to restore the phagocytic function of peritoneal exudate cells and the mitogenic activity of T cells of tumour-bearing mice. Moreover, the induction of gene expression of nine out of seventeen cytokines and five out of six cytokine receptors in peritoneal exudate cells and splenocytes by administration of PSPC prepared from *Tricholoma lobayense* has been confirmed by RT-PCR and *in situ*-hybridization (Liu *et al*., 1996a). This suggests that the immune cells are responding to PSPC through gene expression and the production of immunomodulatory cytokines that might mediate immunopotentiation of this agent *in vivo* (Liu *et al*., 1999).

Immunopotentiation effected by binding of mushroom β-glucans or polysaccharide-protein complexes includes activation of innate defences (such as cytotoxic macrophages, neutrophils and NK cells) and stimulation and proliferation of humoral (B cells) and cell-mediated immune systems (such as helper T cells, promotion of T cell differentiation), and activation of alternative complement pathway. Pharmacologically, these mushroom compounds are classified as biological response modifiers and have antitumour activity, a result of activation or augmentation of the host's immune system or immunocompetency rather than direct

cytotoxicity. However, recent evidence suggests that some mushroom polysaccharides may also possess cytotoxic properties. In search for a more effective treatment for hormone-refractory prostate cancer, the potential antitumour effect of Grifron-D (a unique β-glucan from the Maitake mushroom *Grifola frondosa*) on androgen-independent prostatic cancer PC-3 cells was investigated (Fullerton *et al*., 2000). A dose-response study showed that almost complete (>95%) cell death was attained in 24 h with ≥ 480 μg/ml Grifron-D. Combinations of Grifron-D in a concentration as low as 30 to 60 μg/ml with 200 μM vitamin C were as effective as GD alone at 480 μg/ml, suggesting that vitamin C acts synergistically to potentiate Grifron-D activity. Significantly elevated lipid peroxidation levels and positive *in situ* hybridization staining of Grifron-D treated cells indicated oxidative membrane damage resulting in apoptotic cell death. These seminal findings have shown that this bioactive β-glucan from the Maitake mushroom has a cytotoxic effect, presumably through oxidative stress, on prostatic cancer cells *in vitro*, leading to apoptosis.

The information presented here illustrates the distinct biologic properties associated with mushroom polysaccharides and polysaccharide-bound protein complex immunomodulators. While the activity of some of these polymers has been well-documented, the lack of defined structural and mechanistic information for promising compounds from other mushrooms is limiting efforts to study their potential for clinical use. Recent investigations have led to a more detailed understanding of structural aspects of polysaccharides that influence biologic function and host immune responses (Bohn and BeMiller, 1995). Continued advancements in our understanding of particular structure-function relationships and polysaccharide-specific receptors should provide a foundation for the further development of these compounds that have novel immunomodulatory activities.

In conclusion, a fundamental principal in Oriental medicine is to regulate homeostasis of the whole body and to bring the diseased person to his or her normal state (Chihara *et al*., 1992). Potentiating the physiological constitution in favour of host defence results in the activation of many vitally important cells for the maintenance of homeostasis. We here report that a wide variety of medicinal mushrooms fit the criteria of host defence potentiators where many were shown previously to possess novel characteristics associated with the immune and other systems (such as nervous and endocrine). A variety of polysaccharides from a variety mushrooms have the ability to enhance the immune system, i.e., behave as immunomodulators. All have shown significant anti-tumour activity, a result of activation of the host's immune system, rather that direct cytotoxicity. The most active immunomodulators come from mycelia, fruiting bodies and from culture fluids of fungi and warrant further investigation. The mushroom polysaccharides appear to be well-tolerated and compatible with chemotherapy and radiation therapy. However, studies that identify the molecular mechanisms that occur in specific immuno-modulation by MMs, such as receptors and what downstream events are triggered by the binding of these polymers to their target cells, are urgently needed.

Evidence for β-glucan receptor binding of immune cells

Ross and co-workers (1999) showed recently that β-glucans from fungi bind to specific iC3b-receptors (CR3, CD11b/CD18) of phagocytic cells and NK cells, stimulating phagocytosis and/or cytotoxic degranulation. The iC3b-receptor, CR3, known also as Mac-1 or $\alpha_M\beta_2$-integran, has two major functions. As Mac-1 adhesion molecule, it mediates the diapedesis of leukocytes through the endothelium and it

stimulates phagocytosis and degranulation in response to microorganisms or immune complexes opsonised (i.e., coated with) iC3b (Ross et al., 1999).

Most β-glucan that have immuno-modulatory properties are derived from yeast and fungi (mushrooms) and have a backbone structure of linear β-1, 3-linked D-glucose molecules with β-1, 6-linked sied chains (Bohn and BeMiller, 1995). Although somewhat controversial (Czop and Kay, 1991; Zimmerman et al, 1998), recent data suggest that CR3 serves as the major, if not only receptor for β-glucans with human (Thornton et al, 1996) or mouse (Xia et al., 1999) leukocytes, and therefore, may be responsible for all reported functions of β-glucans *in vitro* and *in vivo*. These β-glucans polymers specifically target macrophages, neutrophils, and NK cells to tumours that are opsonised with antibody and C3 (complement), and therefore, β-glucan appears to have the same specificity as opsonising antibody (Ross, 1999).

As stated by Hobbs (2000) “This research has particularly shown the therapeutic value in mice of small soluble β-glucans (5 – 20 Kda) that bind to CR3 with high affinity and prime, the receptor for subsequent cytotoxic activation if, and only if, CR3 subsequently comes in contact with an iCR3-opsonised target immune cell”. Furthermore, particulate β-glucan and high molecular weight, soluble β-glucans (such as Lentinan and Schizophyllan) that have been used for patient therapy in Japan have been shown to be large enough to cross-link membrane CR3 of neutrophils and monocytes, triggering respiratory bursts, degranulation, and cytokine release (Ross, 2000).

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