Carboxymethylated β-(1-3)-Glucan

A beta glucan from baker's yeast helps protect skin

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Glucans are natural polysaccharides comprised of D-glucopyranosyl units and are found in oat, barley, wheat, microorganisms and fungi. The D-glucopyranosyl units of glucans from cereal grains are linked by β-(1-4) and β-(1-3) bonds, whereas the glucans from yeast and higher fungi consist of a linear β-(1-3) backbone on which occasional (1-6) branching occurs. Most of the applications and safety data have used the linear β-(1-3), yeast-derived glucan.

Glucans from cereal grains are valuable nutritional components of the human diet. Presently, large-scale extractions are being investigated for possible food applications.[31]

Crude extracts from yeast have been used for a long time in cosmetic and pharmaceutical applications. These materials have been found useful in the treatment of many diseases and skin conditions.[11] Glucan preparations from oat and barley have skin-conditioning properties similar to emollients, while glucans containing β-(1-3)-linked glucopyranosyl units are known to have immune-stimulating activity.

In 1941, intensified research on yeast components led to the discovery of Zymosan,[22] the first defined pharmaceutical yeast product with immune-stimulating activity. This product is a raw cell-wall extract composed of glucan, other polysaccharides, proteins and lipids. Further studies have identified β-(1-3)-glucan from the yeast's cell wall as an immunologically effective component. The properties of the yeast-derived glucan are quite similar to those of other biological response modifiers (BRMs), including high-molecular-weight β-(1-3)-glucans derived from fungi. Three of these — schizophyllan, lentinan, and grifolan — have been studied extensively in Japan for their potential efficacy in treating malignant tumors.[21]

Properties of Yeast Glucans

β-(1-3)-Glucan from yeast has been identified as a very potent stimulator of the immune system, with the ability to activate macrophages, neutrophils and other cells that carry specific β-glucan receptors on their surface.[4] Activation of these cells by glucan stimulates the nonspecific defense mechanisms of the host. Other polysaccharides, such as mannans, galactans and α-(1-4)- or β-(1-4)-linked glucose polymers, have no such activity.

Manners et al describe the structure of glucan from baker's yeast (*Saccharomyces cerevisiae*).[18] The alkali-insoluble glucan

Des glucanes carboxyméthylés de la levure fournissent un actif cosmétique hydrosoluble capable de protéger la peau du stress oxydatif induit par UVA. Les glucanes se révèlent aussi capables de stimuler le renouvellement cellulaire et d'améliorer l'immunosuppression provoquée par exposition aux UV.

Carboxymethylierte Glucane aus der Hefe enthalten einen wasserlöslichen kosmetischen Wirkstoff, der die Haut vor dem UVA-bedingtem oxidativen Stress schüzt. Glucane stimulieren die Hauterneuerung und verringern die Immunsuppression bei UV-Strahlung.

Parece que los glucanes también estimulan la renovación cutánea y mejoran la inmunosupresión debida a la exposición al UV.

The Skin's Immune System

The skin is the body's most important primary defense system, both as a physical barrier and as a metabolic and immunological biochemical-response system. At the epidermal level, the skin's immune system involves cytokines and the immunocompetent Langerhans cells (LCs) and keratinocytes (KCs).

LCs: LCs play a dominant role in the immune reaction of the skin and are theorized to be the skin's own version of macrophages. Although they represent only a minor fraction (2-4%) of the cell population, LCs are characterized by dendrites that extend over the whole epidermis. Presumably, one function of this dendrite network is to trap antigens and initiate a general immune response.

The allergen is first captured by the LC and modified before it migrates to the lymphatic system. There, the allergen associated with human leukocyte antigen (HLA) Class II molecules is presented to the specific T-cells (CD4+). The subsequent binding that occurs stimulates the proliferation of the T-cells in the lymph nodes, as well as the production of cytokines, the most important of which are interleukin-2 (IL-2) and interferon-γ (IFN-γ).

KCs: KCs also produce a number of cytokines involved in immune-response regulation and skin inflammation.[23] KCs can produce significant levels of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) upon stimulation from liposaccharides, UV-radiation or chemicals. One unique cytokine produced by KCs is the transforming growth factor-β (TGF-β). This cytokine suppresses most of the immune reactions and may therefore be an important element involved in the down-regulation of immune responses in the skin.

In addition to cytokine production, KCs can also express the major histocompatibility (MHC) genes for HLA-DR. These molecules engulf foreign proteins and present them as antigens to the T-cells. Another important cell surface marker that KCs express is the intercellular adhesion molecule-1 (ICAM-1). The ICAM-1 molecule is crucial to the interactions of the KCs with T-cells and monocytes because it is the ligand for the cell-surface markers on such cells. ICAM-1 is of special interest in allergic contact dermatitis (ACD) because the allergens themselves stimulate its expression.[9]

Immunosuppression responses: Unlike the cytokines, ICAM-1 is not constitutively expressed at low levels by the KCs; instead, it is induced by IFN-γ and TNF-α. The expression of ICAM-1 triggered by IFN-γ has been used to study the protection afforded skin cells by different UV filters against UVB-induced immunosuppression.[5]

Modulation of ICAM-1 expression is not the only immunological response that occurs; UV radiation also alters cytokine release and impairs the number and viability of immunocompetent cells present in the epidermis.[19,24] All such responses constitute examples of immunosuppression. UV-induced immunosuppression has been found to play a crucial role in the development of skin cancer in mice,[7] and it is likely to be implicated as a risk factor in the development of the disease in people.[14,27]

UV protection and immunosuppression: Although there is little dispute that commercially available sunscreen filters inhibit the development of many UV-induced alterations, like erythema,[15] they do not necessarily offer complete protection against other biological effects of sunlight, such as immunosuppression.[8,25] This may have serious consequences: consider the number of people who expose their skin to sunlight for extended periods of time in the belief that the high-SPF sunscreen products they apply will fully protect them.

To compound this problem, the skin becomes even more susceptible to environmental hazards with advancing age, because of a general decline in the body's immune defense mechanisms. The deterioration of this system can be accelerated by certain conditions, one of which is overexposure to the sun. With our increasing awareness of how these additional UV-related interactions and mechanisms can affect the body, concern over UV exposure and the sun-care products we use should also increase. This may change our approach to consumer education and the behavior we adopt in response to this knowledge.

A material that stimulates the immunocompetent cells in the skin could slow the decline of these defense mechanisms. If it also enhances the turnover rate of cross-linked collagen, denatures enzymes and oxidizes extracellular matrix components, this material could significantly improve the skin's general condition. With its well-documented immune-stimulating properties, β-(1-3)-glucan from yeast looks like a very promising candidate.

fraction has a backbone of β-(1-3)-glucopyranosyl units with a low degree of inter- and intramolecular β-(1-6) links.

Researchers have extensively studied the activity of glucan preparations from yeast in wound healing,[26] infectious diseases[16] and oncology.[6,12] In all of these applications, different β-(1-3)-glucan preparations from yeast show activity at low concentrations. Wolk and Danon[26] found a significant acceleration of wound healing in the animal leg model with topical application of a yeast glucan preparation at 20-100 μg/ml.

Because insoluble glucan preparations can produce undesirable toxicological properties, such as granuloma formation, clinical interest has focused on soluble glucan preparations. Recently, a Phase II clinical study showed the tolerability and efficacy of a soluble yeast glucan.[2] Application of a soluble glucan preparation (0.1–2.0 mg/kg) before and after thoracic or abdominal surgery lowered postoperative infection rates. In another investigation, researchers observed improved wound healing following topical administration of a soluble phosphorylated glucan preparation.[32]

Developing a Cosmetic Glucan

Glucan isolated from the cell wall of *Saccharomyces cerevisiae* is a water-insoluble particulate polymer that is

unsuitable for cosmetic applications. We have developed a specialized process that modifies the insoluble pure β-(1-3)-glucan to carboxymethyl glucan (CMG), as seen in Figure 1-1.[a] The carboxymethylation takes place under specific conditions in a reaction that yields a product with an 0.75 degree of substitution.[b] This level of substitution is high enough to render the material water-soluble without disturbing its helical structure, the configuration responsible for its activity. The chemical identity of CMG was confirmed by ^{13}C-NMR spectroscopy.[30]

The carboxymethylated glucan material is both water-soluble, up to 4% concentration, and cosmetically appealing. CMG is compatible with most cosmetic ingredients and tolerates most manufacturing conditions.

The dermatological tolerance of CMG has been determined by carefully monitoring the effects of a 2% aqueous solution on healthy volunteers. CMG did not induce allergic reactions or cause inflammation, confirming that the material is neither an irritant/photoirritant nor a sensitizer/photosensitizer.

Evaluation on Cell Cultures

Because glucan preparations have shown activity at very low concentrations, cell-culture experiments should reveal any activity.

Porcine cells: Researchers at the Frauenhofer Institute (Stuttgart, Germany) investigated the activity of CMG using cultured porcine keratinocytes (KCs). Adding the polysaccharide to the M199 culture medium with 10% calf serum gave a significant, concentration-dependent stimulation of KC proliferation. With 0.01% CMG, the relative cell count increased by more than 35% after 120 hours. However, researchers observed no protective effects against 1 J/cm^3 UVB with 0.01% CMG, regardless if the porcine cell cultures were treated before or after irradiation.[10]

Human cells: CMG was tested for its ability to protect human skin cells against UVA, which is a very potent stimulator of oxidative stress in the epidermis. This stress leads to phototoxic and photoallergic reactions in and on the skin.[1] Like immunosuppression, the effects of oxidative stress induced by UVA appear to be factors in carcinogenesis.[17] The prevention of such stress is, therefore, highly desirable.

Cell cultures of human skin fibroblasts and KCs were developed from normal human skin, samples of which were extracted by biopsy. The cells taken from various donors were pretreated with 0.01% CMG for 18 hours before being exposed to varying doses of UVA (320-450 nm). To demonstrate protection against oxidative stress from CMG pretreatment, researchers measured changes in the intracellular concentration of glutathione (GSH) and ferritin.[29] KCs pretreated with the polysaccharide experienced significantly less depletion of the GSH antioxidant than what normally occurs immediately after UVA radiation. During these experiments, CMG produced effects similar to those of DL-α-tocopherol, an antioxidant used as a control because of its known ability to protect cells from UVA-induced oxidative stress.

[a]Cromoist CM Glucan, Croda Inc.
[b]Patent pending, Mibelle

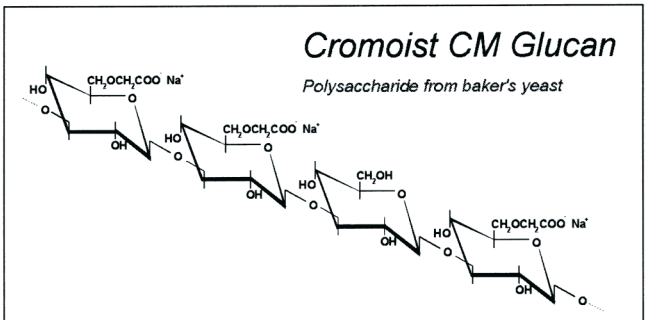

Figure 1-1. Chemical structure of carboxymethylated glucan from baker's yeast. On average, 3 out of 4 glucose units of the β-(1-3)-linked polymer are modified at position 6.

Table 1-1. Test products with CMG concentrations in percent

	Hydrogel	*Emulsion*
Control	0%	0%
Test levels	0.1	0.4, 0.04

Cell-origin effects: The effects observed in cell-culture experiments appear to depend on the cells' origin. For instance, the response to CMG of KCs from pig embryos was not as positive, nor the results as effective, as with the KCs derived from adult human skin.

The immune defense of human skin consists of a complex system of cells. Langerhans cells (LCs) and KCs predominate in the epidermis; T-cells, tissue macrophages and mast cells occur throughout the dermis, and cytokines can be found in both the epidermis and the dermis. Although the cytokines appearing in the two layers are different, their functions — mediating cell communication and regulation — are the same. Given the complexity of this immune defense system, it is likely that the KC culture derived from human skin biopsy is more immunocompetent than the porcine culture. This may result from the increased presence of LCs in the human cell samples or from the expression of different relevant markers by the human KCs.

CMG on Human Skin

To study the efficacy of CMG in vivo, we formulated the yeast polysaccharide into two different types of cosmetic preparations, an oil-in-water (o/w) emulsion and a hydrogel. We evaluated CMG in these systems at concentrations ranging from 0.04 to 0.4%. The test products and two control versions were applied twice daily to the forearm skin of 5 volunteers over a 14-day period (Table 1-1). At the end of the 2-week treatment period, we immersed the skin in a 10% solution of sodium dodecyl sulfate (SDS) for 2 hours in an effort to compromise its barrier function.

Protecting skin moisturization: During the course of product application, treatment with each of the 5 products enhanced skin humidity, measured in corneometer units, as compared to untreated skin. A significantly higher increase in skin humidity occured in skin treated with the o/w emulsion than in skin treated with the hydrogel.

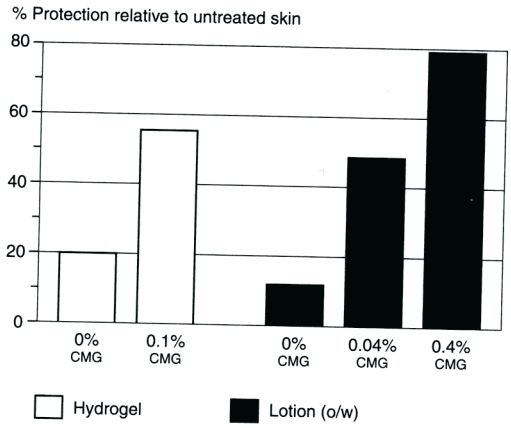

Figure 1-2. Change in transepidermal water loss (TEWL) of skin treated with cosmetic formulations with and without CMG, as measured at Day 14 and after challenge with sodium dodecyl sulfate (SDS). Figures shown are expressed as percentages relative to an untreated control.

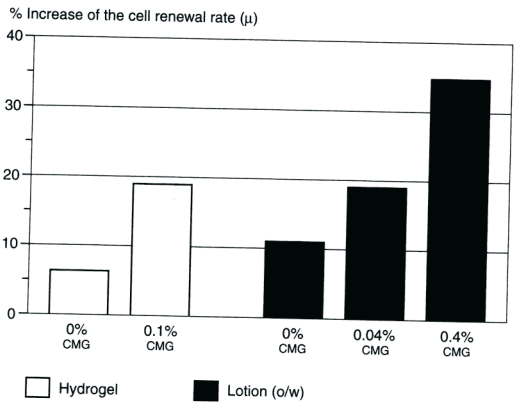

Figure 1-3. Treating skin with preparations containing CMG leads to a concentration-dependent enhancement of the SC cell-renewal rate, calculated as $\mu = I_n\ 2/t_r$, where t_r represents the time it takes the fluorescence of initially applied dansyl chloride to completely diminish.

The subsequent challenge with SDS led to a drastic reduction in skin humidity. However, skin pretreated with the preparations containing CMG showed a much less pronounced decrease in skin humidity, especially in comparison with untreated skin. The strength of this protective effect appears to relate directly to the concentration of CMG in the formulations (Figure 1-2).

Although application of the 5 products had little influence on the rate of transepidermal water loss (TEWL) during the initial 14 days of treatment, we observed a definite increase in TEWL once skin was challenged with SDS and its barrier function damaged. However, we saw much less increase in TEWL vs. the placebo in skin pretreated with the CMG preparations (Figure 1-2). Clearly, the CMG provided a concentration-dependent protective effect.

Enhancing SC renewal: In an additional experiment, we detected an enhancement in the rate of cell renewal in

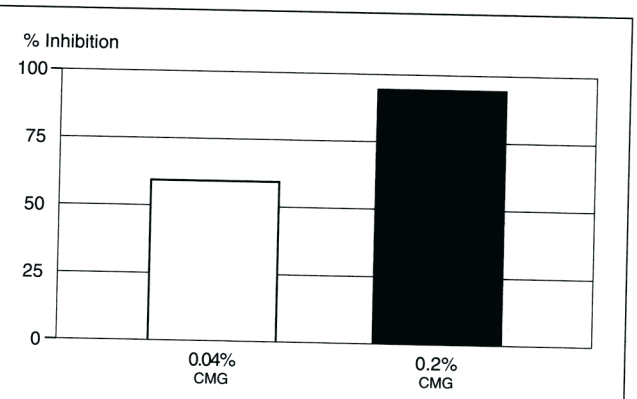

Figure 1-4. In vivo inhibition of squalene peroxidation by CMG, average values of 10 subjects: skin treated with two o/w preparations containing 0.04% and 0.2% of the yeast polysaccharide is protected 59% and 94%, respectively, against peroxidation after UVA exposure; the rate of peroxidation is expressed as the ratio of squalene hydroperoxide to squalene.

skin treated with the CMG preparations. We measured cell proliferation by applying dansyl chloride to skin sites that had been treated with CMG preparations and then determined the rate at which the induced fluorescence diminished. This technique is an established method of measuring cell turnover in the stratum corneum (SC).

The rate of turnover increase clearly depended on the concentration of CMG in the formulation. With 0.4% CMG, the SC experienced more than a 30% increase in cell renewal, compared to untreated skin (Figure 1-3). The increase in cell renewal obtained in vivo correlates well with the increase in cell proliferation found in cultured porcine KCs in vitro.

Inhibiting squalene peroxidation: Squalene is one of the main lipid components of the sebum and is particularly susceptible to photo-oxidation. Colin et al showed that, even with low doses of UVA on the skin, squalene can be converted to squalene hydroperoxide.[3] Free-radical scavengers delivered topically can protect squalene from peroxidation in the skin. To evaluate the in vivo efficacy of CMG in protecting skin against oxidative stress induced by UVA radiation, we used a noninvasive technique for identifying squalene hydroperoxides.

In this particular study, three o/w emulsions containing 0.2%, 0.04% or 0% CMG, were applied to separate sites on the forearm of ten volunteers twice daily for 4 days. On the fifth day, each of the pretreated skin sites and a nontreated site were exposed to 10 J/cm^3 UVA radiation, after which the skin lipids from these irradiated areas were extracted with 1 ml of ethanol. Lipids extracted from an unirradiated area served as a control. We then determined the presence of squalene or squalene hydroperoxide from these extractions using various methods of HPLC analysis.[3,13,20,28]

On examination, all skin extractions contained some concentration of squalene, although the level at which it was present varied widely. Samples taken from skin sites exposed to UVA radiation showed increased squalene hydroperoxide. However, in samples taken from skin sites that had been

pretreated with one of the CMG preparations, the incidence of hydroperoxidation was noticeably reduced (Figure 1-4). As shown here, the use of only 0.04% CMG in the o/w emulsion resulted in a 59% inhibition of the squalene peroxidation. At 0.2%, CMG provided almost complete protection (94%). The protective effect of CMG is expressed here as percent inhibition of peroxidation relative to the placebo. The peroxidation itself is expressed as the ratio of squalene hydroperoxide to squalene.

Such effective protection against UVA-induced lipid peroxidation usually occurs only in the presence of antioxidants. Colin et al reported an inhibition of about 90% upon application of 0.2% D-α-tocopherol.[3] An inhibition of less than 25% was observed with application of the cosmetically stable vitamin E acetate at the same concentration.

From the above data, we conclude that CMG protects skin lipids against oxidation very efficiently and may, in fact, offer additional benefits to UVA-stressed skin that antioxidants do not.

Conclusion

The data we have presented here shows the activity of a carboxymethylated β-(1-3)-glucan from baker's yeast with a degree of substitution sufficient to produce water-solubility. This modified glucan was developed as a biological ingredient for cosmetic and dermatological applications.

- CMG shows activity at very low concentrations — 0.01% — in cell-culture experiments, although the effects observed depend on the type and history of the cells. Keratinocyte cultures developed from pig embryos did not respond as well to CMG as the KCs obtained from adult human skin.
- Results of corresponding in vitro and in vivo experiments correlated well. Pretreating human KCs in vitro with CMG rendered them less sensitive to UVA radiation. The pretreatment of human skin in vivo with cosmetic preparations containing CMG provided substantial protection against the peroxidation of squalene after UVA exposure.
- CMG protects the skin against a decrease in skin humidity and increased TEWL from detergent challenge. To some degree, a second skin effect from the film-forming properties of the polysaccharide may be present. However, the concentrations used in the experiments were too low for CMG to have produced such profound effects solely through its film-forming behavior. Therefore, we speculate that biochemical activation of cells provides much of these protecting effects.

Since CMG is neither an antioxidant nor an iron chelator, it must use mechanisms other than extracellular radical scavenging or activities related to it. CMG appears to stimulate cells, most likely through a receptor-mediated mode, and produce endogenous reaction products that resist oxidative stress and other environmental insults. The triggering of these mechanisms requires CMG to penetrate into the epidermis. (Other negatively charged polysaccharides, like the anticoagulant heparin, can also penetrate skin better than originally thought.) Despite the fact that most polar agents of high molecular weight remain on the skin's surface, CMG does induce biochemical effects within the skin. However, the exact molecular mechanism of the CMG activity has not been fully elucidated and warrants further investigations.

References

1. LA Applegate and E Frank, Variations between individuals against UVA radiation and other oxidative stress in human skin fibroblasts, in *Biological Effects of Light*, Holick and Jung, eds, (1994) pp 71-80
2. TJ Babineau et al, A phase II multicenter, double-blind, randomized, placebo-controlled study of three dosages of an immunomodulator (PPG-glucan) in high-risk surgical patients, *Arch Surg* **129** 1204-1210 (1994)
3. C Colin, B Boussouira, D Bernard and QL Nguyen, Non-invasive methods of evaluation of oxidative stress induced by low doses of ultra violet in humans, A 105, IFSCC Congress, Venice (1994) pp 50-72
4. JK Czop and J Kay, Isolation and characterization of β-glucan receptors on human mononuclear phagocytes, *J Exp Med* **173** 1511-1520 (1991)
5. J Degwert and U Hoppe, Immuno-protection by topical sunscreens, Poster 084, *18th International IFSCC Congress*, Venice (1994).
6. NR Di Luzio, DL Williams, RB McNamee, BF Edwards and A Kitahama, Comparative tumor-inhibitory and anti-bacterial activity of soluble and particulate glucan, *Int J Cancer* **24** 773-779 (1979)
7. MS Fisher and ML Kripke, Supressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice, *Science* **216** 1133-1134 (1982)
8. MS Fisher, JM Menter and I Willis, Ultraviolet radiation-induced suppression of contact hypersensitivity in relation to padimate O and oxybenzone, *J Invest Dermatol* **92** 337-341 (1989)
9. JO Funk and HI Maibach, Horizons in pharmacologic intervention in allergic contact dermatitis, *J Am Acad Dermatol* **31** 999-1014 (1994)
10. T Graeve and A Schneider, Unpublished results
11. R Hänsel, Polysaccharide, Die Immun-stimulierend Wirken: Eine Uebersichet Über Entsprecjende Fertigarzneimittel, *Farmaceutisch Tijdschrift Voor Belgie* **64** 313-326 (1987)
12. M Hofer, M Pospisil, J Bohacek, I Pipalova and J Sandula, Enhancement by carboxymethylglucan of early cellular damage in 1 Gy-irradiated mice, *Folia Biola Praha* **41** 112-117 (1995)
13. AE Holley and TF Slater, Measurement of lipid hydroperoxides in normal human blood-plasma using HPLC-chemiluminescence linked to a diode array detector for measuring conjugated dienes, *Free Radical Res Comm* **15** 51-63 (1991)
14. LF Kinlen, AGR Sheil, J Peto and R Doll, Collaborative United Kingdom-Australian study of cancer in patients treated with immuno-suppresive drugs, *Br J Med* **II** 1461-1466 (1979)
15. LH Kligman, FJ Akin and AM Kligman, Prevention of ultraviolet damage to the dermis of hairless mice by sunscreens, *J Invest Dermatol* **78** 181-189 (1982)
16. PL Kokashis, DL Williams, JA Cook and NR Di Luzio, Increased resistance to *Staphylococcus aureus* infection and enhancement in serum lysozyme activity by glucan, *Science* **199** 1340-1342 (1978)
17. ML Kripke and LA Applegate, Alterations in the immune response by ultraviolet radiation, in *Biochemistry and Physiology of the Skin*, vII Ch 45, L Goldsmith, ed, New York: Oxford University Press (1991) pp 1304-1328
18. DJ Manners, AJ Masson and JC Patterson, The structure of a β-(1-3)-D-glucan from yeast cell walls, *Biochem J* **135** 19-30 (1973)
19. AM Mommaas, AA Mulder and BJ Vermeer, Short-term and long-term UVB-induced immunesuppression in human skin exhibit different ultrastructural features, *Eur J Morphol* **31** 30-34 (1993)
20. HP Nissen and HW Kreysel, The use of HPLC for the determination of lipids in biological materials, *Chromatographia* **30** 686-690 (1990)
21. N Ohno, K Kurachi and T Yadomae, Antitumor activity of a highly branched (1-3)-β-D-Glucan, SSG, obtained from *Sclerotinia sclerotiorum* IFO 9395, *J Pharmacobio Dyn* **10** 478-486 (1987)
22. L Pillemer and EE Ecker, Anticomplementary factor in fresh yeast, *J Biol Chem* **137** 139-142 (1941)

23. S Shimada, The immunological functions of keratinocytes, in *The Biology of the Epidermis,* A Ohkawara and J McGuire, eds, Elsevier Science Publishers BV (1992) pp 203-211
24. A Tang and MC Udey, Doses of ultraviolet radiation that modulate accessory cell activity and ICAM-1 expression are ultimately cytotoxic for murine epidermal Langerhans cells, *J Invest Dermatol* **99** 71S-73S (1992).
25. P Wolf, CK Donawho and ML Kripke, Analysis of the protective effect of different sunscreens on ultraviolet radiation-induced local and systematic suppression of contact hypersensitivity and inflammatory responses in mice, *J Invest Dermatol* **100** 254-259 (1993)
26. M Wolk and D Danon, Promotion of wound healing by yeast glucan evaluated on single animals, *Med Biol* **63** 73-80 (1985)
27. T Yoshikawa et al, Susceptibility of effects of UVB radiation on induction of contact hypersensitivity as a risk factor for skin cancer, *J Invest Dermatol* **95** 530-536 (1990)
28. JR Zhang, AR Cazers, BS Lutzke and ED Hall, HPLC-chemiluminescence and thermospray LC-MS study of hydroperoxides generated by phosphatidylcholine, *Free Radical Biol Med* **18** 1-10 (1995)
29. F Zülli, LA Applegate, E Frenk and F Suter, Photoprotective effects of CM Glucan on cultured human skin cells, *Eurocosmetics*, **11** 46-50 (1995)
30. F Zülli and C Saecker, CM Glucan a new yeast polysaccharide for cosmetic use, *Cosmet Toil Manufacture Worldwide*, 131-136 (1994)

Patents

31. US 5,512,287, L Wang, I Lynch and K Goering, Production of β-glucan and β-glucan product (1996)
32. US 4,833,131, DL Williams and W Browder, Soluble phosphorylated glucan: methods and compositions for wound healing (1989)

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