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Biochemical significance of exogenous chitins and chitosans in animals and patients

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In comparison to other polysaccharides currently used in wound dressing materials, modified chitosans possess outstanding biochemical significance: they promote ordered tissue reconstruction, vascularization, equilibrated presence of cellular elements and little scar formation. An explanation is offered by their susceptibility to the hydrolytic action of lysozyme and *N*-acetyl-β-D-glucosaminidase, which make available chito-oligomers capable of macrophage stimulation and favourable influence on collagen deposition. Oligomeric and monomeric species are also expected to be incorporated into extracellular matrix components which further assist in the rebuilding of physiologically valid tissues.

INTRODUCTION

One century ago, Bruns (1888) and Coley (1891) observed the regression of certain tumours in patients recovering from bacterial infection or intentionally treated with bacterial extracts. In recent years, the attention of numerous researchers has been directed to the immunomodulating properties of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP), a minimal chemical component of glycopeptides of bacterial walls, exhibiting adjuvant activity. One of the properties of MDP is its ability to enhance the cytolytic activity of murine peritoneal macrophages *in vitro* and *in vivo*, with respect to bacteria and tumour cells. A new MDP analogue, *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine, GMDP, which includes the structural unit of chitin in its formula, produced a marked antitumour effect, being more effective than MDP. The mechanism of activation of macrophages exposed to GMDP is based on the depression of 5′-nucleotidase (E.C. 3.1.3.5) activity and the stimulation of production of superoxide radical which appears to be one of the mediators of macrophage cytotoxicity (Balitsky *et al.*, 1989).

Further to studies on the immunological properties of various kinds of bacterial fractions such as the cell-wall skeletons of *Mycobacterium bovis, Nocardia rubra* and *Listeria monocytogenes*, the stimulations of host-defense mechanism against Sendai virus infection in mice was also obtained with chitosan. One day after infection, a higher titre of interferon was detected in the lung washing of mice than those treated with chitosan 1 day before infection (Azuma *et al.*, 1987).

Chitin and chitosan oligomers were studied for their antitumour effects. In particular, water soluble hexa-*N*-acetylchitohexaose, the hexamer of *N*-acetylglucosamine, and chitohexaose, the hexamer of glucosamine were studied against allogeneic and singeneic mouse tumour. Immunotherapeutic agents safer than other complex carbohydrates such as α-D-mannan and lentinan (a β-D-glucan) could be developed because they lack antigenicity and are less prone to accumulation in the host animal. Both hexamers gave complete regression of the solid tumours in mice (K. Suzuki *et al.*, 1986). Besides growth inhibitory effect against solid Meth-A tumours (Tokoro *et al.*, 1988), hexa-*N*-acetylchitohexaose was also found to display antimetastatic effects against Lewis lung carcinoma transplanted into mice, giving rise to a 40–50% inhibition ratio of pulmonary metastasis when administered intravenously. Protection against infection by *Pseudomonas aeruginosa, Listeria monocytogenes* and *Candida albicans* was also noticed in tumour-bearing mice. The *N*-acetylglucosamine hexamer did not show any acute toxicity, mutagenicity, pyrogenicity up to 100 mg/kg body weight and augmented the release of interleukin-1 from macrophages *in vivo*. Interleukin-1, as well as tumour necrosis factor (TNF) are important cytokines

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which activate fibroblasts to produce active proliferation. With regard to the mechanism of action, evidence was provided of the facilitation of the defence functions of macrophages, polymorphonuclear leukocytes, cytotoxic and natural killer cell activity, due to induction of interleukins-1 and -2 (S. Suzuki *et al.*, 1986*a*). Practical applications were envisaged (S. Suzuki *et al.*, 1986*b*; Shiratori *et al.*, 1988).

Chitosan was found to be the most effective immunomodulator for the activation of non-specific host resistance against bacterial viral infections and tumour growth in mice. This effect was partly mediated by stimulating cytocydal activities of peritoneal macrophages and natural killer cells (Nishinuma, 1992). Intraperitoneally administered chitosan augmented superoxide generation from peritoneal exudate macrophages in mice (Hishimuna *et al.*, 1990). Moreover, chitosan stimulated antigen-specific immune responses such as the production of antibody to bacterial α-amylase, induction of delayed hypersensitivity to azo dyes, and induction of alloreactive cytotoxic T lymphocytes in guinea pigs and mice.

The effect of chitosan on the production of cytokines by peritoneal macrophages and by spleen cells was determined (Nishimura, 1986). The peritoneal activated macrophages induced stimulation of natural killer cells (lymphocyte-like cells which are known to kill spontaneously and aspecifically an ample variety of tumour cells and virus-infected cells) and of helper T cells, which secrete lymphokines, whose release was actually enhanced by chitosan. It is important to recall that helper T cells do not specifically react with the antigen. Chitosan did not affect the ability of spleen cells to produce lymphokines. Thus, chitosan showed immunomodulating effect mainly by stimulating the production of monokines (K. Nishimura *et al.*, 1986*a*); carboxymethyl chitin was less efficient (K. Nishimura *et al.*, 1985, 1986*b*).

Glucosamine takes part in detoxification functions of liver and kidneys and possesses anti-inflammatory, hepatoprotective, antireactive, and antihypoxic activities (Yakovleva *et al.*, 1988; Zupanets *et al.*, 1990; Setnikar *et al.*, 1991*a*). Works on the therapy of arthrosis led to commercial use of glucosamine sulphate (Setnikar *et al.*, 1991*b*). Exogenous glucosamine stimulates synthesis of proteoglycans, exerts trophic action on joint cartilages and favours sulphate ester formation in chondroitin sulphate. Such activities counterbalance degeneration of cartilage; all toxicity and mutagenesis tests were negative.

Incorporation of glucosamine in glycosaminoglycans was also demonstrated: for instance, rats were injected intraperitoneally with D-[^{3}H]-glucosamine which was incorporated into the cochlea and into the articular cartilage, particularly into hyaluronan (Kleine & Bowman, 1990; Gil-Loyzaga *et al.*, 1991). Incorporation of labelled glucosamine into psoriatic epidermis was less than into normal epidermis (King *et al.*, 1981). D-Glucosamine has been shown to have natural killer activity in human patients. Unlike most other antitumour drugs, D-glucosamine is selectively toxic to in-vitro tumours, producing tumour necrosis with little or no damage to normal tissues (Matheson *et al.*, 1984).

CHITINS AND CHITOSANS IN WOUND MANAGEMENT

From the data presented in the previous section, convincing evidence is obtained that chitin and certain derivatives can be safely applied to animals as well as to the human body; even more, they exert a number of beneficial actions when the human body is affected by some of the worst diseases. The Japanese Ministry of Health and Welfare approved chitosan as an ingredient for hair care products in 1986, and carboxymethyl chitin for skin care products in 1987; the chitin nonwoven fabric was approved as a skin substitute in 1988. Chitosan was also admitted as a food additive.

Biocompatibility of chitosan administered orally and intravenously was assessed in animals, LD_{50} being over 16 g/kg in mice, i.e. higher than for sucrose. No consequences were detected in rabbits, broilers and hens. Hexosamine value was 11 μmol/g blood in rabbits after administering low molecular weight chitosan (3000 D) intravenously at a dosage of 4·5 mg/kg *pro die* and went back to normal level after discontinuing the intravenous administration (Hirano *et al.*, 1988). In-vivo tests for blood compatibility were carried out by the same authors; while chitosan exhibits heamostatic activity, particularly when it is used as a solid of high molecular weight, water-soluble depolymerised chitosan and oligomers have no thrombogenic activity. It has been indicated that chitosan has an ability to form a coagulum upon contact with erythrocytes even in heparinized blood, defibrinated blood and washed red blood cells. Chitosan may prove to be useful as an aid to heamostasis in patients with coagulopathies. Lingual bleeding times were sharply decreased in rabbits without apparent systemic effects (Malette *et al.*, 1983; Klokkevold *et al.*, 1991). The action of chitosan on blood could be modulated by various chemical modifications leading to *N*-acylchitosans (Hirano *et al.*, 1987*b*) or sulphate esters (Muzzarelli *et al.*, 1984; Muzzarelli & Giacomelli, 1987) which are not only heparin-like substances but also antiviral products (Gama Sosa *et al.*, 1991).

Absorbable sutures were proposed, based on high molecular weight chitin, which could exert heamostatic action and undergo slow degradation by lysozyme (Nakajima *et al.*, 1986; Singhal *et al.*, 1988). Evidence of the lysozyme attack *in vivo* and *in vitro* was provided by electron microscopy according to Nakajima *et al.*

(1986). Multi-porous beads were also tested in particular for macrophage activation (S.I. Nishimura *et al.*, 1986), which actually could be confirmed as cytotoxicity for cancer cells, and stimulation of interleukin-1 production.

Chitin-based products for wound dressing today include: regenerated chitin powders, chitin non-woven fabrics, porous beads, lyophilized soft fleeces, gel-forming lyophilized soft fleeces and gauzes, laminated sheets, double-face laminated sheets, transparent films, microspheres, and associations with other polymers such as cellulose, collagen, keratin, chondroitin sulphate, polyester, poly(tetrafluoroethylene) and polyurethane.

Non-woven fabrics made of fibres of either chitin or chitosan were recently developed for use as wound dressings (Oshima *et al.*, 1987). Chitin solutions in dimethylacetamide + LiCl were used for spinning 6 μm fibres, which were cut to various lengths and sedimented to form a thin mat. After γ-ray sterilization, such chitin dressings were used on patients mainly to heal burns. These preparations are presently commercially available in Japan. This regenerated chitin, once applied to a wound, is slowly hydrolysed and disappears after 3 months (Kifune, 1992). Regenerated chitosan has been developed into fibres, films, porous beads and microspheres. The highest susceptibility to lysozyme was found for chitosans having a degree of deacetylation of 0·70, and no susceptibility was found for fully deacetylated chitosans, therefore the former should be preferred for medical applications. As far as the immunoadjuvant activity is concerned, chitosan was found to be endowed with the same properties indicated above for chitin. The metabolism of regenerated chitosans was investigated after oral and subcutaneous administration of ^{125}I-labelled chitosan microspheres to mice. A remarkable accumulation of the radioisotope was observed in both instances around the intestinal system and stomach; relatively rapid metabolism was observed in liver and spleen. Surgical lesions of liver in rabbits were covered with regenerated chitosan membranes (10 μm thick), which exerted heamostatic activity by suppressing postsurgical bleeding and reducing secretion of plasminogen activator by macrophages (Seo, 1992). Composite films were also proposed for manufacturing adhesive bandages for oral surgery. Such bandages consist of an insoluble lining layer and an adhesive layer made of poly(acrylic acid) and chitosan in a ratio between 5/95 and 70/30. Chitosan acetate fibres (diameter 1 μm, length 500 μm) were added to poly(acrylic acid) in ethanol and the resultant mix was spread on the lining layer (Itoi *et al.*, 1985; Yoneto *et al.*, 1990). Chitosan bandages could also be prepared by laminating a porous felt made by freeze-drying a chitosan solution together with a chitosan film made by evaporating the same chitosan solution (Takagi *et al.*, 1990*a*, *b*). A reconstructed skin from co-cultured human keratinocytes and fibroblasts on a chitosan-collagen-chondroitin sulfate was proposed by Shahabeddin *et al.* (1991) and used to treat burned patients.

APPLICATIONS TO LABORATORY ANIMALS

Most significant experiments were done in vertebrates (monkeys, dogs, cats, calves) (Minami *et al.*, 1992). Debridement of wound and rapid formation of granulating tissue, as well as proliferation of fibroblasts and vascularization are indispensable aspects in the mechanism of healing of a wound in mammals. Research works were conducted to examine the effect of chitin as an activator of macrophages. Chitin was used in the form of a chitin sponge, chitin flakes and polyester non-woven fabric containing chitin particles. Such remedies were used for implant of vulnus cavity or prosthesis of partial tissue deficit of tendon and of intrapelvic connective tissue after the reduction of perineal hernia.

In the chitin-treated animals, wounds healed by first intention in all cases (against 50% in controls). Activated macrophages and fresh neutrophils were more abundant than in controls. On the fourth day, fibroblast activation was also intense. In no case were suppuration and microbial proliferation reported. The organisation of the polyester non-woven fabric within the connective tissue was more evident in the chitin group, and by the 18th postoperative day, vascularisation with medium size arteries became evident. Mitosis of fibroblasts was frequently observed on the second/fourth day as a point of difference from controls. For most (80%) of the animals treated with the chitin non-woven fabric, a single treatment was sufficient.

In the case of chitin sponge, 83% of the animals healed by formation of granulation tissue by a single treatment. Especially for face bite wounds (cats and monkeys) complete healing within 2 weeks was reached with a single treatment.

HISTOLOGICAL RESULTS WITH ANIMALS AND PATIENTS

Skin tissue repair is sustained by fibroblast proliferation, collagen deposition, angiogenesis and subsequent re-epithelialisation. During the wound healing process, cells produce such compounds as fibronectin, glycosaminoglycans, collagen, laminin and proteoglycans which are components of the extracellular matrix. All of these compounds, secreted into the extracellular space, are believed to provide a structural scaffolding and a suitable microenvironment to cells undergoing morphogenesis.

The multiplication of fibroblasts, which characterizes wound repair morphologically, is stimulated by substances released by platelets and macrophages. Fibroblasts then secrete collagen, initially immature, but able to provide vital structural support to fragile newly-formed capillaries.

The scar tissue is a particular connective tissue, frequently poor in elastic fibres, with a tendency to retract. In humans, contraction stops at an earlier stage than in rodents, with a greater amount of scar tissue separating the wound edges, resulting in retracting scars and functional impairment. In the late stages of the normal process of wound healing when collagen synthesis declines and high oxygen tension is no longer required, many new vascular channels regress: the wound becomes usually avascular and undergoes a transformation into a scar with limited tissue elasticity. In surface lesions, the rebuilding of normal tissue is made possible by the basal cells of the epidermis, which proliferate and cause the tissue to regenerate, but, for lesions which penetrate deeply into the dermis, this process becomes more difficult because the layer of basal cells is lost, and tissue rebuilding is entrusted only to cells bordering the injured area. This process, however, is very slow and frequently interrupted by infections.

In laboratory animals, *N*-carboxybutyl chitosan has been demonstrated to induce formation of ordered repair tissue containing collagen bundles regularly arranged (Muzzarelli *et al.*, 1988). *N*-Carboxybutyl chitosan gels favoured the formation of loose connective tissue, thus facilitating diffusion of substances and cell proliferation. Vascular structures were present with good histoarchitectural order of the stromal matrix. On the 30th day, complete re-epithelialisation was observed with all the epithelial layers represented. Underlying connective tissue was mature but looser than for controls. Very limited inflammatory processes and absence of suppurative complications were further favourable aspects (Muzzarelli *et al.*, 1990*a*, *c*). Wounds treated with *N*-carboxybutyl chitosan did not show evident scars or wound contraction; rather, *N*-carboxybutyl chitosan favoured angiogenesis and, most important, prevented the regression that the wound usually undergoes from capillary rich to avascular scar.

Donor sites, in patients undergoing plastic surgery, were treated with soft pads of freeze-dried *N*-carboxybutyl chitosan to promote ordered tissue regeneration. Compared to control donor sites, better histoarchitectural order, better vascularisation and absence of inflammatory cells were observed at the dermal level, whilst fewer aspects of proliferation of the malpighian layer were reported at the epidermal level.

Regression of angiogenesis took place in any case as soon as chitosan was no longer administered or had been absorbed; nevertheless, the resulting connective tissue appeared orderly structured and endowed with good functionality (Biagini *et al.*, 1991*b*). The *N*-carboxybutyl chitosan provided a tridimensional supporting lattice favouring the epithelial cell migration, and, in a way, modulated re-epithelialisation.

Capsule formation is another aspect of importance in plastic surgery. Anomalous deposition of connective tissues takes place when the dynamic equilibrium between synthesis and breakdown is altered, leading to fibrosis, a ubiquitous, aspecific and disordered increase of collagen. The fibrous capsule formed after implanting a tissue expander under the skin is a macroscopic aspect of such a reparative process. The connective tissue cellular components responsible for the organisation of the collagen lattice determine such a structure by exerting oriented traction forces. Steps leading to capsular structure are: (1) mesenchymal elements are attracted and concentrated where the traction is stronger, and increase it; (2) fibroblasts aligned along the major axis of the extracellular fibre bundles tend to orientate the fibres along that axis, thus amplifying the process of structural orientation.

Silicone expanders coated with *N*-carboxybutyl chitosan were inserted into surgical wounds in the dorsal skin of rabbits. During all of the steps of capsular organisation, *N*-carboxybutyl chitosan sustained correct proliferation and organisation, and stimulated physiologically the tissue repair process; angiogenesis was favoured while fibrogenesis was depressed. The formation of vascularized connective tissue with copious mesenchymal elements and reduced collagen component indicated the ability of *N*-carboxybutyl chitosan to assist newly formed tissues in retaining good trophicity and loose state, which are favourable physiological characteristics. *N*-Carboxybutyl chitosan increased the interfibrillar amorphous substance in the dermal region close to the expander and reduced the damages generated by the foreign body. The loose capsular tissue formed in its presence was less prone to contraction/retraction during maturation (Biagini *et al.*, 1991*a*).

The well-known difficulties in obtaining spontaneous repair of the meniscal structure in orthopaedics are a real challenge to the use of biomaterials intended for promotion of a guided repair of the cartilaginous tissue.

The angiogenetic properties of *N*-carboxybutyl chitosan assume particular importance in the repair process of the meniscus. In fact, such repair is conditioned by the presence of vessels that chitosan could be able to extend from adjacent capsular structures. In rabbits, results indicated that *N*-carboxybutyl chitosan was well tolerated at the articular-synovial level. It also favoured and stimulated the repair processes which do not take place spontaneously in the meniscus.

In the meniscal areas close to the synovial lining, the angiogenetic stimulus provided by *N*-carboxybutyl

chitosan led to further repair processes of the meniscal tissue. Observations made on the synovium 45 days after application of *N*-carboxybutyl chitosan showed that the synovial membrane had cells layered on subintimal stromal tissue exhibiting tightly packed collagen fibres among which mesenchymal cells and vascular structures were visible. The meniscal tissue was characterised by structural reparative aspects, with irregularly distributed collagen bundles, evolving towards cartilaginous tissue, microvessels were also present. It was therefore concluded that healing of certain meniscal lesions can be promoted by *N*-carboxybutyl chitosan (Muzzarelli *et al.*, 1992*b*).

In ageing skin, collagen undergoes cross-linking reactions and physicochemical alterations; proteoglycans decrease in general and their percentage ratios are altered. Vascular walls undergo thickness increase and a reduced quantity of oxygen reaches the cutaneous tissue with unfavourable consequences in the case of ulcers and burns. Hypertension, oedema, atherosclerosis and diabetes further reduce the quantity of oxygen, nutrients and cells having defensive action (leukocytes and macrophages) reaching the cutaneous tissue.

Aged patients suffering from leg ulcers were submitted to treatment with *N*-carboxybutyl chitosan fleeces or gels, for periods of time up to 30 days. Compared to controls, a more rapid epithelialization was noted (7 days instead of 15–20 days). In no case did infections occur and good hemostasis was observed. From the morphological point of view, controls showed the usual disordered deposition of collagen fibres, whilst in patients treated with *N*-carboxybutyl chitosan, a correct histoarchitecture of the regenerated skin was observed, in particular an ordered organisation and vascularisation of the derma (Muzzarelli, 1992).

Urological operations are often unsuccessful because excessive fibrosis occurs in the wound, especially in tubular structures of the urogenital system. Renal wounds produce extensive destruction of surrounding kidney parenchyma because the fibrosis of healing obstructs distal tubules, thus renal damage would be reduced if fibrosis could be minimised.

A preliminary study, designed to examine surgically induced wounds of the kidney, ureter and penile foreskin in dogs, answered questions about the feasibility and safety of the application of chitosan to urogenital wounds. Chitosan solutions, which exhibited a marked immediate heamostatic effect, could be applied easily to urogenital wounds without modification of the usual surgical technique. No infections occurred and no foreign body granulomas were encountered. Despite the spillage on surrounding tissues, few peritoneal adhesions were encountered. In ureters, chitosan demonstrated early healing, with evidence of tissue reapproximation, and ingrowth of capillaries was most prominent at 4 days. In kidneys, minimal fibrous reaction was noted, and a decrease of fibroplasia was evident. In foreskin, the ingrowth of fibroblasts was decreased by chitosan. The use of chitosan, therefore, could minimize fibrosis and reduce obstruction (Bartone & Adickes, 1988).

Periodontitis is a kind of infectious disease caused by specific bacteria, leading to serious destruction of periodontal tissues. The periodontal ligament plays structural and functional roles as a hard-soft tissue interface. Migration, adherence, proliferation and differentiation of periodontal ligament cells are essential functions for periodontium regeneration. Potent chemotactic and mitogenic activities on periodontal ligament cells were reported for *N*-acetyl-D-hexosamines, extracellular matrix, polypeptide growth factors and *O*-carboxymethyl chitin, chitosan and low molecular weight phosphorylated chitin, both *in vitro* and in rodents. *O*-Carboxymethyl chitin showed potent chemotactic activity and collagen gel contractability; it had no effect on cell proliferation. Alkaline phosphatase activity was not reduced. Differences in biological function between modified α- and β-chitins were also reported. Low molecular weight phosphorylated chitosan showed the highest chemotactic activity, collagen gel contractability and it did not lower alkaline phosphatase activity (Katsuragi, 1992).

Chitosan ascorbate, obtained by mixing chitosan with ascorbic acid and sodium ascorbate, was produced in a gel form suitable for the treatment of periodontitis according to current dental surgery. Chitosan was progressively reabsorbed with very satisfactory clinical recoveries of the 52 defects treated, for which tooth mobility and socket depth were significantly reduced (Muzzarelli *et al.*, 1989).

Associations of chitosan with poly(tetrafluoroethylene) were proposed to delay the apical migration of the epithelium to prevent formation of periodontal sockets and to give the periodontal ligament enough time to rebuild the tissue in contact with the dental surface (Sakai, 1987). Chitosan-coated microvascular prostheses improved healing (Van Der Ley & Wildevuur, 1989). Chitosan-bonded hydroxyapatite bone filling paste was proposed as a bone substitute (Sumita, 1989; Ito, 1991); chitosan imparted better biocompatibility, favoured cell migration and inhibited adsorption of oral streptococci on two hydroxyapatites (Sano *et al.*, 1987).

In view of the carie-preventing effect of chitosan oligomers (Shibasaki *et al.*, 1988), due to pH control, heamostatic action and bacteriostatic action (Itoi *et al.*, 1987), dentrifrices for plaque inhibition and denture cleansers were formulated with chitin derivatives (Komiyama *et al.*, 1984; Komiyama & Hitoi, 1988). As for the expected action on bone formation based on an increase in alkaline phosphatase activity, work is now in progress (Namita *et al.*, 1988; Muzzarelli, 1993).

Osteoconduction promoted by modified chitosans was observed by Muzzarelli *et al.* (1992*a*) subsequent to the avulsion of widsom teeth.

Results *in vitro* showed that *N*-carboxybutyl chitosan is particularly active against Gram-positive bacteria including *Streptococcus* spp., *Staphylococcus aureus, Staphylococcus epidermidis* and *Staphylococcus haemolyticus*. Observations by the electron microscope showed that the cell wall had been affected by the presence of the water-soluble chitosan, which provided fraying and weakening. Bacterial duplication was possibly depressed. Data showing bacteriostatic-bactericidal action were also obtained for *Pseudomonas, Escherichia, Proteus, Klebsiella, Serratia, Acinetobacter, Enterobacter* and *Citrobacter* spp. (Muzzarelli, 1990; Muzzarelli *et al.*, 1990*b*). The candidacidal action was also assessed.

SUSCEPTIBILITY TO LYSOZYME AND *N*-ACETYLGLUCOSAMINIDASE

The occurrence of lysozyme (E.C. 3.2.1.17) in the human body has been studied widely; this muramidase was found to occur in nose, bronchus, bronchiole, middle ear, lacrimal gland, bone marrow and in the digestive tract (Pinkus & Said, 1977; Franken *et al.*, 1989). Lysozyme is present in serum (Porstmann *et al.*, 1989; Giles *et al.*, 1990; Harada *et al.*, 1990), saliva (Pajari *et al.*, 1989; Konttinen *et al.*, 1990) and other fluids (Portsmann *et al.*, 1989) including those surrounding the cartilage (Thonar *et al.*, 1988).

Activation of macrophages is thought to be regulated by signals encountered in the tissue microenvironment during their development from monocyte precursors. Those local signals include the cytokines interferon-γ and tumor necrosis factor-α (Lewis *et al.*, 1990). Monocytes and macrophages have a remarkable secretory repertoire with most products being released only in response to extracellular signals; by contrast, lysozyme and fibronectin are secreted continuously by macrophages even in the absence of exogenous stimulation (Gordon *et al.*, 1974; Lewis *et al.*, 1989, 1990).

Hirano *et al.* (1991) reported an enhancement of serum lysozyme activity upon injection of a mixture of chitosan oligosaccharides intravenously in rabbits. No abnormal physiological symptom was observed in the rabbits during and after the intravenous injection at a dosage of 4·5 mg/kg of body weight per day for 11 days. The serum hexosamine value increased to 11·6 μmol/ml serum on the second day after the last day of injection, and decreased to 5·5–7·7 μmol/ml on the 13th day (Table 1).

The normal value of lysozyme activity in rabbit serum was 4·4 ± 2 U/ml. As shown in Table 2, the activity was enhanced to 9·2 after 5 injections of chitosan oligosaccharides at a dosage of 7·1–8·6 mg/kg per day, and the high activity lasted for about 5 days after the last injection. However, the lysozyme activity was neither enhanced by injecting chitin oligosaccharides, galactosaminan oligosaccharides and saline, nor

Table 1. Fate of chitosan oligosaccharides, low molecular weight chitosan and galactosaminan oligosaccharides in rabbit serum subsequent to intravenous injection (adapted from Hirano *et al.*, 1991)

Substance injected[a]	Hexosamine (μmol/ml of serum) Day after the last injection		
	2nd	5th	13th
Control		5·4–7·7	
Chitosan oligosaccharides	10·2 ± 0·4	n.d.	5·5 ± 0·2
low-molecular-weight	11·6 ± 0·2	9·6 ± 0·9	7·7 ± 0·3
Galactosaminan oligosaccharides	7·9 ± 1·9	5·4 ± 0·7	6·0 ± 0·4

[a]Injected at a dosage of 4·5 mg/kg of body weight for 5 days into rabbits weighing 2·0–2·6 kg.
n.d. = not determined.

Table 2. Enhancement of serum lysozyme activity by intravenous injection of chitosan oligosaccharides, chitin oligosaccharides or galactosaminan oligosaccharides in rabbits (adapted from Hirano *et al.*, 1991)

Substance injected[a]	Blood lysozyme activity (U/ml of serum) Day after the last injection				
	1st	3rd	5th	7th	60th
Saline (control)	4·4 ± 1·2	4·4 ± 2·0	n.d.	n.d.	4·3 ± 1·2
Chitosan oligosaccharides	9·2 ± 2·2	7·7 ± 2·2	6·9 ± 2·4	3·7 ± 1·0	4·7 ± 2·0
Chitin oligosaccharides	4·4 ± 1·5	3·7 ± 1·6	n.d.	n.d.	n.d.
Galactosaminan oligosaccharides	4·5 ± 0·9	4·4 ± 0·8	n.d.	n.d.	n.d.

[a]Injected at a dosage of 7·1–8·6 mg/kg body weight per day for 5 days into rabbits weighing 3·5–4·2 kg.
n.d. = not determined.

enhanced by adding chitosan oligosaccharides into a medium for an in-vitro culture of whole rabbit blood. These data obtained by Hirano *et al.* (1991) indicated that lysozyme is introduced into blood by chitosan oligosaccharides from specific cells of blood vessels or organs.

It is also important to note that human monocyte-derived macrophages can be stimulated by human interferon-γ to release *N*-acetyl-β-D-glucosaminidase isoenzymes (Bourbouze *et al.*, 1991), which would be able to carry further the depolymerisation process to the monomeric units. While a major consequence of the administration of chitin and derivatives is the immunoadjuvant effect, a so far underestimated consequence is the incorporation of the hydrolysis products into normal components of the connective tissues.

Chitins, chitosans and their derivatives so far proposed for medical use are susceptible to lysozyme hydrolysis. Besides the wealth of information on this topic in the field of the elucidation of the structure of lysozyme and in the field of chitosan-based excipients for delayed release of drugs (outside the scope of this study), most of the literature cited here contains scattered information on the in-vivo action of lysozyme on chitin, chitosan and some derivatives. More works on lysozyme catalysed depolymerisation of chitin/chitosan could be mentioned (Fukamizo *et al.*, 1986; Sahiwa *et al.*, 1990; Fukamizo & Goto, 1991). Current research on susceptibility of chitosans to depolymerisation under the catalytic action exerted by lysozyme is confined to relationships between molecular size or degree of deacetylation and rate of hydrolysis (Nakano *et al.*, 1987; Hirano *et al.*, 1989; Yomota *et al.*, 1990). Works were also done on chitin by Muzzarelli (1977, 1985, 1988, 1989); Muzzarelli & Pariser (1978); Muzzarelli *et al.* (1986, 1990*a*); Zikakis (1984); as far as modified chitins are concerned, carboxymethyl chitin and dihydroxypropyl chitin in the form of fibres were exposed to lysozyme under controlled conditions (Tokura *et al.*, 1983) and improved accessibility was found, compared to plain chitin. Kinetic data on *N*-substituted chitosans, such as *N*-carboxybutyl chitosan, indicate that they exhibit high susceptibility to lysozyme (Muzzarelli, 1993). These are peculiar features of modified chitins and chitosans: in fact, acetamidodeoxycellulose and aminodeoxycellulose, semisynthetic analogues of chitin and chitosan, respectively, were not hydrolysed by lysozyme and chitinases (Hirano *et al.*, 1987*a*). The nature of the substrate clearly has a major influence on its susceptibility to lysozyme attack. Nascent chitins and colloidal chitins are most readily attacked. Highly ordered ('crystalline') chitins and bleached chitins are most resistant.

Lysozymes act as endohydrolases and catalyse hydrolysis of (1-4)β linkages. Related enzymes are *N*-acetyl-β-D-glucosaminidases, E.C. 3.2.1.30, some of which can hydrolyse the terminal non-reducing *N*-acetylglucosamine residue of chitin. For review articles on their chemistry and occurrence in the human body, see Proctor & Cunningham (1988) and Muzzarelli (1993).

Glucosidase activity can also result in the formation of new glycosidic bonds, by transglycosylation. This reaction has been investigated for hen egg-white lysozyme, where the rate constant for transglycosylation is much larger than for hydrolysis (Chipman, 1971; Tadu & Kakitani, 1973; Masaki *et al.*, 1981). Transglycosylation seems to be favoured by high substrate and salt concentrations, and was described as follows:

$$GGGG^* + E = GGE + GG^*$$

$$GGGG^* + GGE = GGGGGG^* + E$$

(E = enzyme, G = *N*-acetylglucosamine unit, * = reducing end).

It is possible that in some situations *in vivo*, the transglycosylation activities of lysozyme may be their major roles, rather than their lytic activities.

The hydrolytic actions of lysozyme and *N*-acetyl-β-D-glucosaminidases, which make available chito-oligomers capable not only of macrophage stimulation and favourable influence on collagen deposition, but also of being incorporated into extracellular matrix components (Fig. 1), are therefore the key factors which explain the activities exerted by chitosans in the rebuilding of physiologically valid tissues.

CONCLUSIONS

Regenerated chitins and chitosans and their chemically modified derivatives combine in themselves biological significance, convenient physicochemical characteristics, in-vivo biodegradability, biocompatibility, antimicrobial action and, in the healing wound, behave as controlled delivery sources of *N*-acetylglucosamine, D-glucosamine and substituted glucosamines. While, so far, it has not been definitely assessed, this information is apparent from the cited literature. Most recently, in postoperative peritoneal healing, the incorporation of glucosamine by exudative and tissue repair cells was reported (Orita *et al.*, 1991). Several explanations for a relationship between glycosaminoglycan production and collagen synthesis have been offered. It has been suggested that the glycosaminoglycan component of the extracellular matrix may participate in modulating the deposition of collagen (Bertolami, 1984). Although fibroblasts are known to synthesize both collagen and glycosaminoglycans, different mechanisms for controlling the production of

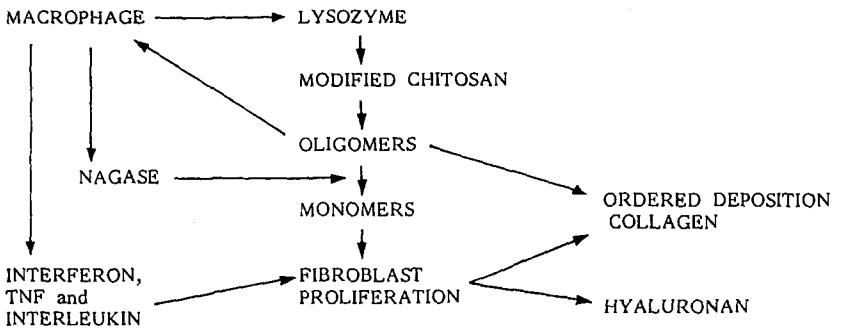

Fig. 1. Lysozyme, normally produced by macrophages, hydrolyses susceptible modified chitosan to oligomers which activate macrophages to produce interferon, TNF and interleukin-1. Activated macrophages increase their production of lysozyme and also produce *N*-acetyl-β-D-glucosaminidases which catalyse production of D-glucosamine, *N*-acetylglucosamine and substituted glucosamines from oligomers. These aminosugars are available to fibroblasts which proliferate under the action of interleukin-1, for incorporation into hyaluronan thus guiding the ordered deposition of collagen, also influenced by chito-oligomers.

each have been demonstrated (Green & Goldberg, 1983; Green & Hamerman, 1984).

The mechanism of immunoadjuvant activity of chitosan is also based on lysozyme. Lysozyme-digested chitosan can penetrate easily into the cells. Amplification of humoral response via macrophage, interleukin-1, interleukin-6 and lymphocytes may be the mechanism of adjuvant activity (Marcinkiewicz *et al.*, 1991).

The induction of lysozyme into blood by chitosan oligosaccharides is essentially similar to the induction of lysozyme in insects, and chitinases in plants. These processes for the body defensive functions are followed by the activation of the macrophage system in animals and by production of phytoalexins in plants and antibacterial protein in insects.

The present study, therefore, indicates that oligomers and monomers generated by lysozyme action on exogenous chitosan-based medical items are utilized for rebuilding connective tissue components. Under certain conditions, chitin-based dressings are in part transformed into structural components of the connective tissues, and therefore a further aspect of their biological and biochemical significance is brought to light, i.e. their incorporation by tissue repair cells and by extracellular matrix components. This permits us to understand the uniqueness and peculiarity of action of chitin-based wound dressings, compared to similar items made of other polysaccharides, proteins or man-made polymers.

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