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Biocompatibility of Wound Management Products: A Study of the Effects of Various Polysaccharides on Murine L929 Fibroblast Proliferation and Macrophage Respiratory Burst

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Abstract—An in-vitro screening method to examine the biocompatibility of materials used in wound management has been evaluated. This involved the use of a macrophage respiratory-burst assay and a fibroblast proliferation assay to represent respectively the inflammatory and the granulation phases in wound healing. Standard polysaccharides (calcium and sodium alginates, *ι*-carrageenan, chitin, chitosan lactate, chondroitin sulphate and pectic acid) were used as test compounds. None of the polysaccharide samples caused a significant increase in L929 fibroblast cell numbers relative to control after 6 days incubation. The overall effect of exposure of the fibroblast cultures to the alginates, carrageenan and chondroitin sulphate was an extension of lag phase followed by an enhanced rate of cell proliferation in the logarithmic phase. Only calcium and sodium alginates and chondroitin sulphate enhanced the respiratory burst activity of murine macrophages; *ι*-carrageenan and chitosan lactate were markedly inhibitory. The results suggest that a macrophage activity assay should be included as part of an in-vitro screening program to evaluate the biocompatibility of wound management materials and to detect intrinsic biological activity.

Upon tissue injury, the coagulum which forms (containing platelets, fibrin, thrombin, chemotactic factors and growth factors released by platelets) increases capillary permeability and dilatation, and attracts macrophages and leucocytes to the wound and hence initiates the acute inflammatory phase (Peacock 1984; Bucknall & Ellis 1984; Burton 1986; Leaper 1986; Wahl 1988a). Macrophages participate in the cleansing process of the damaged tissue and are also responsible for initiating angiogenesis and the appearance of fibroblasts through the action of the growth factors or cytokines they release such as interleukin-1, platelet derived growth factor, fibroblast growth factor, tumour necrosis factor, fibroblast-activating factor, and fibroblast-activating peptide (Wahl 1988b). In the second phase of wound healing, granulation tissue (fibroblasts and ground substance which consists mostly of glucosaminoglycans) fills the dead space and matures. Collagen is actively synthesized by the fibroblasts, deposited into the extracellular matrix and then cross-linked to give tensile strength to the newly-healed wound (Peacock 1984; Bucknall & Ellis 1984). It has been suggested that the macrophage deactivating factor released by fibroblasts suppresses the macrophage respiratory burst and perhaps serves to limit tissue damage during the late stages of the inflammatory response (Szuro-Sudol & Nathan 1982; Nathan & Tsunawaki 1986).

A wide variety of materials has been used in wound healing and the range of dressings purportedly promoting wound healing is increasing. The use of many of these is based on anecdotal claims or non-comparative open studies (Leaper 1986). Usually the efficacy of a wound management product is determined either by clinical assessment, guided by empirical observations, or through the use of wound models.

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However, these procedures give only minimal information on the local cellular response as described above. The further development of these materials requires a better understanding of their effects on the qualitative and quantitative aspects of the cellular response of the two major phases of wound healing. A fibroblast culture method to determine biocompatibility of both existing and potential wound management materials has been developed in our laboratory (Schmidt et al 1989; Turner et al 1989). We have now attempted to evaluate a macrophage activity assay for possible inclusion into our screening protocol.

Materials and Methods

Cell line

L929 mouse fibroblasts were obtained from ICN Flow, High Wycombe, UK.

Culture media

Unless stated otherwise, all cell culture materials were supplied by ICN Flow, High Wycombe, UK.

Complete growth medium for L929 mouse fibroblasts consisted of Minimum Essential Medium Eagle (Modified) with Earle's salts (EMEM) containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) supplemented with non-essential amino acids (1% v/v), 2 mM L-glutamine, 100 units mL^{-1} penicillin, 100 $\mu g\ mL^{-1}$ streptomycin, and 10% newborn bovine serum.

Earle's balanced salt solution (modified) without calcium and magnesium containing trypsin (0·25% w/v) was used in cell trypsinization procedures.

Cell counting was carried out using an improved Neubauer haemacytometer chamber (BDH) as previously described (Turner et al 1989).

Routine maintenance of the cultures

Stock L929 cells were stored in liquid nitrogen. New cultures were initiated from frozen stock; cells were grown in sterile 75 cm^2 culture flasks in complete EMEM medium at 37°C. Cultures were passaged weekly and media changed three times weekly. L929 cells in their 573rd and 580th passages were used in this work.

Chemicals

Calcium/sodium alginates were supplied by Courtaulds Research, Coventry, UK. The calcium content of the alginates was determined by atomic absorption spectroscopy. Pectic acid was obtained from Aldrich Chemical Co., Gillingham, Dorset, UK. Chitosan lactate (Sea Cure+210 Lactate) was obtained from Protan Ltd, Drammen, Norway. Chitin (purified powder from crab shells), ι-carrageenan (Type V, from *Eucheuma spinosa*, containing approx. 2·7% Ca, 2·4% K, and 1·9% Na), chondroitin sulphate A (sodium salt from bovine trachea, containing approx. 30% chondroitin sulphate C), and other reagents were obtained from Sigma Co. Ltd, Gillingham, Dorset, UK.

Preparation of polysaccharides

Chondroitin sulphate dissolved in the complete medium was filter sterilized through a 0·2 μm Millipore filter. All other polysaccharides were sterilized with 70% v/v ethanol and allowed to dry in a laminar flow cabinet before adding the culture medium. The pH was adjusted to 7·2–7·3 with sterile 1 M sodium hydroxide when necessary.

Cell proliferation study

On day 0, 5×10^5 cells in 5 mL of complete EMEM medium were seeded into each 25 cm^2 flask and placed in an incubator at 37°C. After 24 h (day 1), the spent medium in the flasks was replaced with freshly prepared medium (5 mL) containing polysaccharides. Identical control cultures in medium not supplemented with polysaccharides were also prepared. On day 3, the spent medium in the cultures was replaced with the appropriate fresh medium (7·5 mL).

Haemacytometer counts were taken on days 1, 3 and 6. Three flasks from both test and control cultures were counted on each occasion as described previously (Turner et al 1989). Cell viability was estimated using the trypan blue exclusion test (Freshney 1986).

Murine macrophage respiratory burst assay

Quiescent macrophages were harvested from the peritoneal cavity of female WSP mice (aged 6–12 weeks, killed by cervical dislocation) by washing the cavity with 3 mL Eagle's medium containing 0·5% newborn bovine serum. Cells from several mice were pooled and centrifuged at 50 *g* to remove platelets. The number of nucleated cells in the lavage was determined by mixing an aliquot with white cell diluting fluid, consisting of 1% w/v gentian violet and 2% v/v acetic acid in 0·9% NaCl (saline), at a dilution of 1 in 10, using a white cell pipette. Cells were then counted using a haemacytometer. The relative percentage of macrophages in the lavage was calculated using the α-napthylacetate esterase method (Stuart et al 1973). The percentage of macrophages varied between 50–56%. Before use, the lavage was diluted to $1{\cdot}5\text{–}2{\cdot}5 \times 10^7$ macrophages mL^{-1}.

The respiratory-burst assay was adapted from the method of Baehner & Nathan (1968). A sterilized sample of polysaccharide was added (0·1% w/v) to 1·0 mL of the macrophage suspension which was then incubated at 37°C for 30 min. Continuous agitation of the suspension was necessary to prevent the cells from attaching to the sample in the case of the insoluble polysaccharides. Macrophages pre-incubated in the absence of polysaccharide were used in controls.

Control and test solutions consisted of balanced salt solution (ICN Flow) (0·35 mL), 0·01 M potassium cyanide (0·1 mL), 0·85% w/v NaCl (0·4 mL) containing 0·1% w/v nitroblue tetrazolium chloride NaCl solution, and latex beads (polystyrene, 0·8 μm diameter, 0·05 mL). Solutions were pre-warmed at 37°C for 15 min in centrifuge tubes before an aliquot (0·1 mL) of the macrophage suspension pre-incubated either in the presence or absence of polysaccharides (as described above) was added and the tubes incubated for a further 30 min. The reaction was then terminated by adding 0·5 M hydrochloric acid (10 mL) and the tubes centrifuged (1000 *g*, 15 min) at 4°C. The supernatant was aspirated and the 1000 *g* pellet extracted with pyridine (2 mL) for 10 min in a boiling water bath. The tubes were then recentrifuged (500 *g*, 10 min) and the pyridine removed. The pellet was extracted a second time with fresh pyridine (2 mL) and the two pyridine extracts pooled. The optical density of the resulting nitroblue formazan in pyridine solution was determined at 515 nm. The procedure was repeated in triplicate for each polysaccharide and for the controls. The polysaccharide-stimulated respiratory-burst activity of macrophages was calculated as shown below, the results being expressed as a percentage increase or decrease in absorbance at 515 nm induced by the test material relative to that induced by latex particles alone (control).

$$\text{Polysaccharide-stimulated respiratory-burst activity} = \frac{A_{515\,(\text{Test})} - A_{515\,(\text{Control})}}{A_{515\,(\text{Control})}} \times 100$$

Results and Discussion

With the exception of the calcium alginate containing 9·79% Ca which apparently failed to affect the growth of L929 cells,

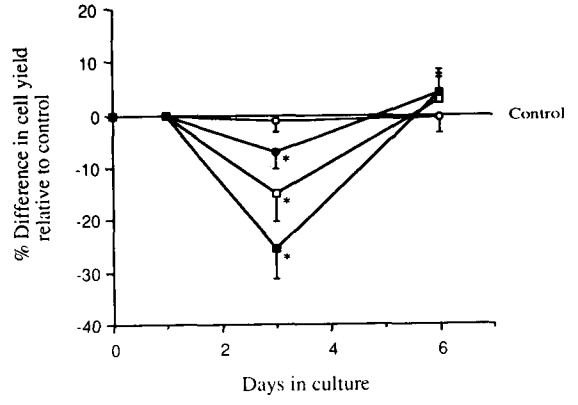

FIG. 1. Percentage difference in yield of L929 cells relative to control on days 1, 3 and 6 in the presence of 0·1% w/v calcium/sodium alginates. ○ 9·79, ● 8·14, □ 5·77 and ■ 3·14% calcium content. Values are mean ± s.d. (n = 3), $^*P < 0{\cdot}05$ compared with control (Student's *t*-test).

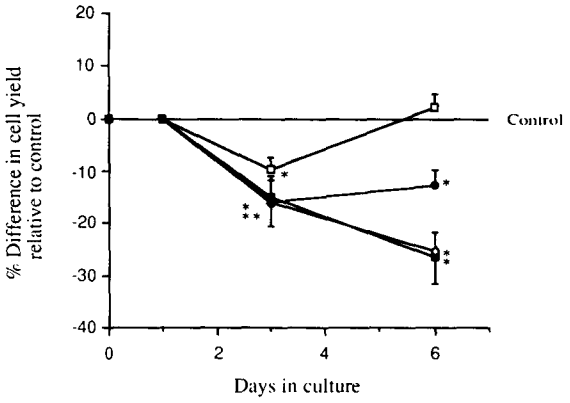

FIG. 2. Percentage difference in yield of L929 cells relative to control on days 1, 3 and 6 in the presence of 0·1% w/v carrageenan (●), chitin (○), chondroitin sulphate (□), and pectic acid (■). Values are mean ± s.d. (n = 3), $*P < 0{\cdot}05$ compared with control (Student's *t*-test).

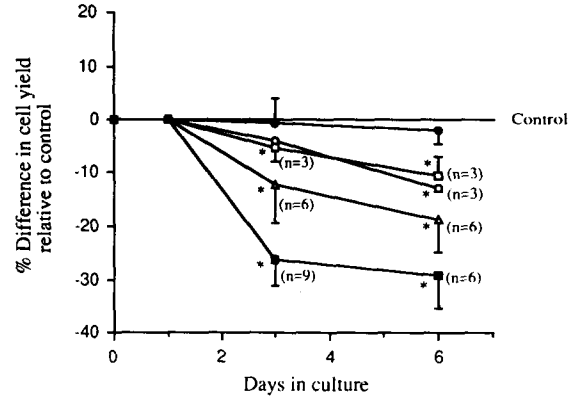

FIG. 3. Percentage difference in yield of L929 cells relative to control on days 1, 3 and 6 in the presence of chitosan lactate 0·01 (■), 0·05 (○), 0·10 (□), 0·50 (●) and 1·0 (△)% w/v. Values are mean ± s.d. $*P < 0{\cdot}05$ compared with control (Student's *t*-test).

all cultures supplemented with alginates showed a significant ($P < 0{\cdot}05$) reduction in cell yield on day 3 followed by a recovery to control levels by day 6 (Fig. 1). Moreover, the greatest decrease in cell yield on day 3 and the most rapid recovery to control levels at day 6 occurred with the alginate having the lowest calcium content. The reduction in the rate of cell proliferation at day 3 did not appear to be a consequence of cytotoxicity since cell viability was high, as determined by trypan blue exclusion, and since cell number on day 3 ($8–20 \times 10^5$ cells/5 mL) was greater than the initial seeding density (5×10^5 cells/5 mL). These results may be viewed in terms of effects on the lag and logarithmic phases of the growth curve; the lower the calcium content of the alginate, the greater the time spent in lag phase and the greater the subsequent rate of growth in the logarithmic phase. Chondroitin sulphate showed a similar effect whilst *ι*-carrageenan, chitin and pectic acid all produced marked decreases in cell yield relative to control by day 6 (Fig. 2). These polysaccharides were also non-cytotoxic at the concentration tested as determined both by trypan blue exclusion and by comparison of cell numbers with initial seeding density. Fig. 3 shows how L929 cells respond to increasing concentrations (0·01–1·0% w/v) of chitosan lactate. Whilst the lowest concentration tested caused a significant reduction ($P < 0{\cdot}05$) in the rate of cell proliferation relative to control at both day 3 and day 6, increasing concentrations appeared to have a progressively smaller effect up to 0·5% w/v whilst 1·0% w/v again caused a marked reduction in the rate of cell proliferation. These observations remain unexplained.

Our results with chondroitin sulphate and carrageenan are essentially identical to previously reported findings. Schjeide et al (1989) found that chondroitin-4-sulphate enhanced the rate of human fibroblast cell proliferation but concluded that it was not a mitogen but that it seemed to provide an enhanced metabolic environment. Tveter-Gallagher et al (1982) observed that *ι*-carrageenans did not affect the logarithmic phase of cell proliferation but did prolong the lag phase relative to control cultures. There do not appear to have been any similar studies of the effect of pectin, chitosan or chitin on fibroblast proliferation reported in the literature, but a previous study with a calcium alginate wound management product has demonstrated a small pro-proliferative effect in L929 cultures counted after 7 days (Schmidt et al 1986).

Both the enhanced rate of cell proliferation following the initial extension of lag phase, as observed with some of the polysaccharides, and the inhibition of cell proliferation as observed with others can be rationalized by reference to

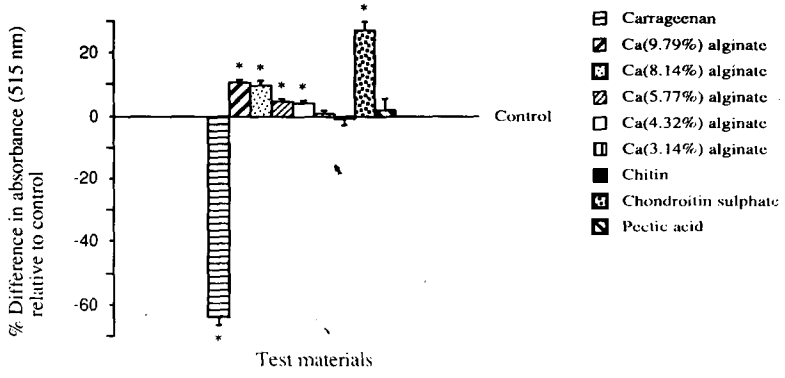

FIG. 4. The effect of various polysaccharides (0·1% w/v) in the murine macrophage respiratory burst assay expressed as percentage differences in absorbance at 515 nm. (Positive values indicate a level of release of superoxide by macrophages that is greater than control.) Values are mean ± s.d. (n = 3), $*P < 0{\cdot}05$ compared with control (Student's *t*-test).

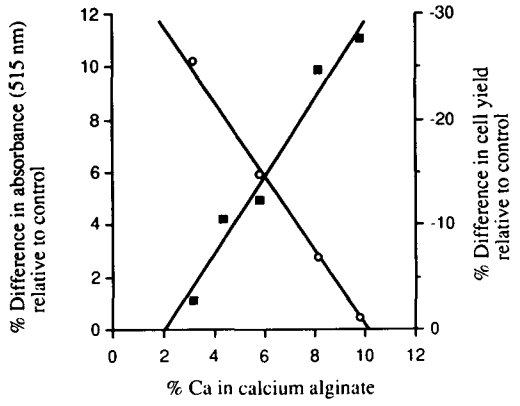

FIG. 5. Positive and negative correlation between the amount of calcium in the various calcium/sodium alginates and the effect of the alginates in the murine macrophage respiratory-burst assay and the fibroblast proliferation assay (on day 3), respectively. ■ Absorbance, O cell yield.

existing literature. The polyanionic polysaccharides will undoubtedly be capable of binding metal ions (other than calcium or sodium) present in the culture medium; chitin, though not polyanionic, is also known to bind metal ions (Muzzarelli 1973; Bell 1977). Most significantly, an ability to bind traces of iron may lead to the autoxidation of the polymers in the medium, with the result that some superoxide and hydrogen peroxide will be formed (Miller et al 1990). This will generate oxidative stress which will to some extent be counteracted by the antioxidant properties of the polysaccharide itself. Iron alginate, for example, is known to autoxidize rapidly (Smidsrød, personal communication) whilst seaweed alginate has been reported to exhibit antioxidant properties (Learn et al 1987). Hydrogen peroxide is known to enhance the rate of L929 cell proliferation when present in micro- to nanomolar concentrations (Schmidt et al 1992). It is also known that the activity of plasma membrane Ca^{2+}-ATPases may be inhibited by oxidative stress and this, in turn, leads to a sustained rise in intracellular free Ca^{2+} as calcium ions present in the culture medium continue to enter the cell down their electrochemical gradient and as bound calcium is mobilized from intracellular stores (Nicotera et al 1985; Hebbel et al 1986; Orrenius et al 1989). These events can be expected to cause an increase in the rate of cell proliferation following the activation of Ca^{2+}-dependent protein kinases (Morgan 1989). More profound oxidative stress or intracellular calcium accumulation will cause inhibition of cell proliferation and, if severe, cell death (Orrenius et al 1989).

Our results from the murine macrophage respiratory-burst assay showed significantly enhanced superoxide generation only following exposure to calcium alginates and chondroitin sulphate (Fig. 4). *ι*-Carrageenan proved to be markedly inhibitory in this assay, a paradoxical result in view of the use of carrageenan in the rat paw oedema assay, a standard model of an inflammatory reaction. It appeared to have been efficiently taken up by the macrophages but evidently inhibited their respiratory-burst activity. Our findings are in general agreement with literature reports that carrageenans are rapidly taken up by macrophage but that this leads to impairment of macrophage function (Di Rosa 1972; Yung & Cudkowicz 1978). As in the case of the effect of the calcium alginates on fibroblast proliferation, the respiratory-burst activity of macrophages appeared to be linearly correlated with the calcium content (Fig. 5), indicating perhaps that calcium ions were responsible for the observed effect. The data depicted in Fig. 5 suggest the calcium content in the alginates has to be above 2% (intercept on the x-axis) before there is any stimulated macrophage respiratory-burst activity. This result is consistent with the observations that calcium influx promotes a variety of responses in macrophages. These include activation of cell locomotion, secretion of hydrolytic enzymes, and increase in oxidative burst and endocytosis (Schneider et al 1979, 1982; Pantaleo et al 1982). The effect of chondroitin sulphate on the respiratory-burst activity would appear to be mediated through a different mechanism. The effect of pectic acid was slightly

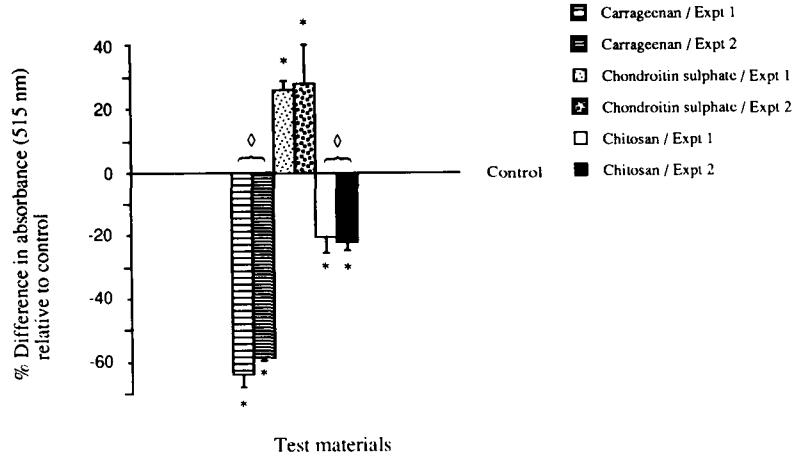

FIG. 6. Comparison of the effects of carrageenan, chondroitin sulphate, and chitosan lactate (all 0·1% w/v) on the murine macrophage respiratory burst. Results from two experiments each carried out in triplicate for analysis of variance. Values are mean ± s.d. (n = 3), $*P < 0.05$ compared with control (Student's *t*-test). ◇ No significant difference between means ($P > 0.05$).

greater than the effect of the 3·14% calcium alginate whilst the effect of chitin was hardly different from control.

Chitin has also been demonstrated to have no effect on mouse peritoneal macrophages in terms of cytolytic activity and interleukin-1 production (Nishimura et al 1986a, b). If the chitin is 70% deacetylated or 6-*O*-carboxymethylated, however, macrophage activity appears to be greatly enhanced (Muzzarelli et al 1988; Nishimura et al 1984, 1986a, b, c).

The macrophage assay is intrinsically problematic because of its reliance on the activity of macrophages which themselves exhibit wide variability in their state of activation. We therefore evaluated the reproducibility of the assay using ι-carrageenan, chondroitin sulphate, and chitosan lactate. Fig. 6 shows the results from two experiments (each carried out in triplicate). All three polysaccharides produced results that were significantly different from control ($P < 0.05$). Analysis of variance showed that except in the case of chondroitin sulphate, there was no significant difference between means from each of the two experiments ($P > 0.05$). In the case of chondroitin sulphate, two apparently spurious results in one of the experiments contributed to the high value for variance, there being a significant difference between means at the 95% level but not at the 99% level. Since the purpose of the assay is to screen materials for activity rather than to obtain absolute measures of activity, we considered the occurrence of occasional spurious results to be acceptable.

In conclusion, it is evident that a macrophage activity assay representing the inflammatory phase of wound healing should be included in an in-vitro screening programme evaluating the biocompatibility of materials used in wound management.

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