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ORIGINAL ARTICLE

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Selective inhibition of cell proliferation and DNA synthesis by the polysulphated carbohydrate ι-carrageenan

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Abstract ι-Carrageenan is a polysulphated carbohydrate that antagonises some heparin-binding growth factors. We assessed the effect of ι-carrageenan on the proliferation of a panel of cell lines, some of which require heparin-binding growth factors for mitogenesis. The importance of growth factor antagonism for the anti-proliferative activity was also determined. Cell proliferation was determined by cell counts and a tetrazolium dye (MTT) assay, and DNA synthesis was determined by thymidine incorporation. The proliferation of the basic fibroblast growth factor (bFGF)-dependent endothelial cell line FBHE was inhibited by daily administration of ι-carrageenan in a dose-dependent manner [concentration inhibiting cell growth by 50% (IC_{50} value), approx. 0.5 μg/ml]. However, excess bFGF did not reverse the inhibitory effect. DNA synthesis was completely inhibited by concentrations of ι-carrageenan that nonetheless allowed significant protein synthesis to occur. The proliferation of the androgen-dependent prostate-carcinoma cell line LNCaP was also inhibited by ι-carrageenan (IC_{50} value, 5.5 μg/ml) and the cells were arrested at the G1/S boundary. ι-Carrageenan inhibited DNA synthesis in MCF-7 cells stimulated by bFGF and transforming growth factor α (TGFα) but not in those stimulated by insulin-like growth factor 1 (IGF-1). Blocking IGF-1-mediated DNA synthesis with anti-IGF-1 receptor antibody αIR3 enhanced the inhibitory activity of ι-carrageenan against MCF-7 cells grown in serum. A number of other transformed and non-transformed cell lines were either partially inhibited or not inhibited by ι-carrageenan. ι-Carrageenan had low anti-coagulant activity. ι-Carrageenan is a selective anti-proliferative agent and warrants further investigation for anti-angiogenic therapy (in view of its activity against endothelial cells) and for the treatment of androgen-dependent prostate cancer.

Key words ι-Carrageenan · Growth factors

Introduction

Carrageenans are polysulphated polygalactans isolated from red seaweeds and are used in the food industry as stabilising agents. There are several forms of carrageenans and these differ from one another in their content of 3,6 anhydro-D-galactose and D-galactose and in the number and position of ester sulphate groups [1] (see Table 1). Carrageenans have a number of physiological effects, such as modulation of normal immune functions [2]. Anti-metastatic activity has been demonstrated for λ-carrageenan (a highly sulphated form) against rat mammary adenocarcinoma 13762 MAT cells [3], possibly due to inhibition of tumour-cell-derived heparanases [4]. κ-Carrageenan and ι-carrageenan have low toxicity when injected intraperitoneally in mice, but the toxicity of λ-carrageenan is higher [5].

Recently, we have shown that carrageenans inhibit the binding of some growth factors [6]. Several lines of evidence suggest that carrageenans have a selective affinity for heparin-binding growth factors. Firstly, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and transforming growth factor β (TGFβ), which bind heparin, were inhibited by carrageenans, whereas transforming growth factor α (TGFα) and insulin-like growth factor 1 (IGF-1), which do not bind heparin, were not inhibited [6]. In addition, heparin competes with ι-carrageenan for inhibition of binding of bFGF, indicating that ι-carrageenan binds to the heparin-binding domain on this growth factor [7].

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Since carrageenans antagonise some heparin-binding growth factors, they may have anti-proliferative activity against cells that require heparin-binding growth factors for mitogenesis. Many tumour cells respond mitogenically to heparin-binding growth factors; examples include glioma cells stimulated by bFGF and PDGF [8, 9], prostate cancer cells stimulated by bFGF [10], breast cancer lines stimulated by pleiotrophin [11] and breast cancer lines and lung carcinoma stimulated by the heparin-binding ligand of c-erbB-2 [12, 13]. bFGF is also implicated in the tumourigenesis of U87MG and T98G glioma cell lines [8], and over-expression of aFGF and bFGF was found in a recent study to correlate with tumour stage in human pancreatic cancer [14]. In addition to a direct mitogenic action on tumour cells, heparin-binding growth factors may also act indirectly by modulating tumour growth via stromal cells or capillary endothelial cells (paracrine regulation). Paracrine stimulation of capillary endothelial cell proliferation is required for the neo-vascularization of tumours and this process of angiogenesis is essential for the sustained growth of tumours [15]. Many angiogenic agents bind heparin [16]. Thus, antagonists of heparin-binding growth factors have the potential to exert an anti-tumour effect either directly by inhibiting tumour cells or indirectly by inhibiting angiogenesis.

Several polysulphated carbohydrates that antagonise heparin-binding growth factors and have anti-proliferative activity have been described. Pentosan polysulphate (PPS) is a potent inhibitor of endothelial cell proliferation and excess bFGF reverses this anti-proliferative activity, suggesting that growth-factor antagonism is the mechanism of action of this compound [17]. PPS inhibits the growth of a number of tumours grown as xenografts in nude mice but does not inhibit the growth of the tumour cells in vitro [18]. Thus, PPS probably exerts its in vivo anti-tumour activity via inhibition of angiogenesis [18]. Heparin, itself a polysulphated carbohydrate, also inhibits some cell lines [19]. However, heparin has numerous effects on cells and growth factor antagonism is probably not the primary mechanism of action of this compound [20].

In this report, we describe a preliminary investigation of the anti-proliferative activities of carrageenans, in particular *ι*-carrageenan, against a number of tumourigenic and non-tumourigenic cell lines. In addition, we evaluate the anti-coagulant activity of carrageenans, as this is a problem associated with the in vivo use of some polysulphated carbohydrates [21].

Materials and methods

Chemicals

Carrageenans (Sigma, Poole, UK) were made up fresh as 2-mg/ml solutions in 25 m*M* HEPES, (pH 7.4) previously heated to 60°C. The properties of the carrageenans used in this study are shown in Table 1. Cell-culture media were obtained from Gibco BRL (Paisley, Scotland). Growth factors were supplied by Bachem (Saffron Walden, UK). Monoclonal mouse anti-IGF-1 receptor antibody αIR3 was obtained from Cambridge Bioscience (Cambridge, UK). Heparin (H3514) and other reagents were supplied by Sigma.

Cell culture

BEAS-2B cells were a kind gift of Dr. C.C. Harris (National Cancer Institute, Bethesda, Md., USA). All other cell lines were purchased from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). L23/P, L23/R, MOR, BEN, LUDLU1, T47D and LNCaP cells were maintained in RPMI/10% foetal calf serum (FCS). BEAS-2B, U87MG and MCF-7 cells were maintained in DMEM/10% FCS. Mouse Swiss 3T3 cells were maintained in DMEM/10% newborn calf serum. PC-3 cells were maintained in Ham's F12/10% FCS, 1% amino acids, 1% non-essential amino acids. Bovine FBHE cells were maintained in DMEM/10% FCS and supplied with bFGF (10 ng/ml every other day). All media were supplemented with antibiotics (100 units penicillin/ml, 100 μg streptomycin/ml). All cell lines are of human origin unless otherwise stated.

Table 1 Properties of carrageenans (*cat.* Catalogue., *Gal* galctose)

	Carrageenans		
Properties Source	κ *Eucheuma cottonii*	*ι* *E. spinosa*	λ *Gigartina aciculaire, G. pistillata*
Sigma cat. number	C1263	C 4014	C3889
Sulphate (%)	24.0	32.5	43.2
3,6-Anhydro-D-galactose	26.0	20.4	0
Galactose (%)	31.9	27.2	48.2
Sulphate groups per disaccharide unit	1	2	3
Position of sulphate groups(s)	β-1,3-D-Gal-4-SO_4/3,6-anhydro-α-1,4-D-Gal	β-1,3-D-Gal-4-SO_4/3,6-anhydro-α-1,4-D-Gal-2-SO_4	β-1,3-D-Gal-2-SO_4/α-1,4-D-Gal-2,6-diSO_4
Solubility in cold water	Na^+salt-soluble	Na^+ salt-soluble	All salt-soluble
Gelation	Gels most strongly with K^+	Gels most strongly with Ca^{2+}	Non-gelling

Proliferation experiments

All experiments were carried out in heat-inactivated serum (56°C, 1 h). This prevented precipitation by ι-carrageenan of serum proteins, which we observed with some batches of serum. Cells were used during the exponential growth phase. For DNA synthesis determinations, cells were plated in 96-well microtitre plates (L23/P, 2000 cells/well; BEN, LUDLU1, 8000 cells/well; all other lines, 4000 cells/well) for 1–2 days depending on the cell line. Fresh medium containing carrageenan was then added and 24 h later the incorporation of [methyl ^{3}H]-thymidine (0.5 μCi/well) over a 2 to 6-h period into trichloroacetic acid (TCA)-insoluble material was determined [22]. For cell proliferation, FBHE cells were plated at 1000 cells/well in DMEM/10% FCS containing 10 ng bFGF/ml. The following day, fresh medium containing carrageenan was added. Adherent cells were trypsinised and trypan-blue-negative cells were counted with a haemocytometer. For tetrazolium dye (MTT) determination, cells were plated down over-night in 96-well microtitre plates (2000 cells/well, except for L23/P, 1000 cells/well; and BEN and LUDLU1, 4000 cells/well). Fresh medium containing carrageenan was then added and the numbers of viable cells were determined by an MTT assay 5 days later [23].

Growth-factor stimulation

MCF-7 cells (10^4/ml) were plated down for 2 days in DMEM/10% FCS. The cells were washed in serum-free DMEM and incubated in serum-free DMEM containing transferrin (10 μg/ml) and BSA (0.2 mg/ml; defined medium) for 2 days as previously described [24]. ι-Carrageenan and growth factor (10 ng/ml) in defined medium were then added and DNA synthesis was determined 24 h later

Protein synthesis

FBHE cells were plated over-night at 4000 cells/well. Fresh medium containing carrageenan was then added and 24 h later the incorporation of L-[4, 5 ^{3}H]-leucine (1.0 μCi/well) over a 2-h period into TCA-insoluble material was determined [22].

Cell-cycle analysis

LNCaP cells were grown to about 50% confluence. Fresh medium containing ι-carrageenan was then added and cells were harvested at intervals by trypsinisation and resuspended in complete medium. Cells were treated with ethidium bromide (400 μg/ml) in 1% Triton X-100 and ribonuclease A (0.5 mg/ml) and analysed on a flow cytometer.

Anti-coagulant-activity assay.

Carrageenan (diluted with 0.9% NaCl, 10 μl) was mixed with pooled plasma (90 μl) and the coagulation time was measured with a KC-10 coagulometer (Amelung, Lemgo, Germany). The activated partial thromboplastin time (APTT) was determined with Pathrombin (Behring, Marburg, Germany).

Results

Comparison of carrageenans

Initially, we compared the anti-proliferative activities of ι, κ- and λ-carrageenans, the three commercially available types from Sigma. Preliminary experiments indicated that for a number of cell lines, κ-carrageenan had weak anti-proliferative activity as compared with ι- and λ-carrageenans (data not shown). λ-Carrageenan had high anti-coagulant activity, whereas the anti-coagulant activities of κ-carrageenan and ι-carrageenan were low (Fig. 1). This report therefore mainly describes the anti-proliferative activity of ι-carrageenan.

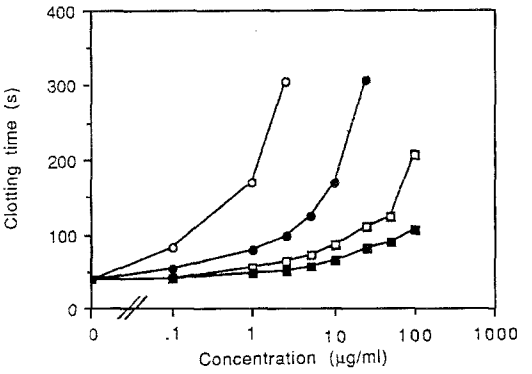


Fig. 1 Anti-coagulant activities of carrageenans in an APTT assay. heparin (○), ι-carrageenan (■), κ-carrageenan (□) and λ-carrageenan (●).

Proliferation of FBHE cells

Since ι-carrageenan is a potent inhibitor of bFGF binding (see Introduction), we investigated the effect of this agent on the bFGF-dependent endothelial cell line FBHE. Figure 2 shows the effect of ι-carrageenan on the number of viable FBHE cells remaining attached to microtitre wells. FBHE cell numbers increased exponentially in the presence of bFGF (Fig. 2a). There was also a slight increase in cells grown in the absence of bFGF (Fig. 2b). This may have been due to residual bFGF from the initial plating medium, which contained bFGF to allow the cells to attach. Proliferation of FBHE cells was partially suppressed by 1 μg ι-carrageenan/ml and completely suppressed by higher concentrations. Non-viable (trypan-blue-positive) cells constituted < 10% of the adherent cells for all concentrations of ι-carrageenan. The majority of non-adherent cells were trypan-blue-positive (data not shown). Fig. 2c shows a dose-response curve. In medium supplemented with bFGF, ι-carrageenan inhibited FBHE cells with a dose of about 0.5 μg/ml, causing 50% inhibition of cell proliferation (IC_{50}). The small increase in cell numbers in the absence of exogenous bFGF was inhibited by low doses of ι-carrageenan.

DNA synthesis and protein synthesis of FBHE cells

There was 30% inhibition of DNA synthesis at 6 h after treatment with 3 or 10 μg ι-carrageenan/ml (Fig. 3a).

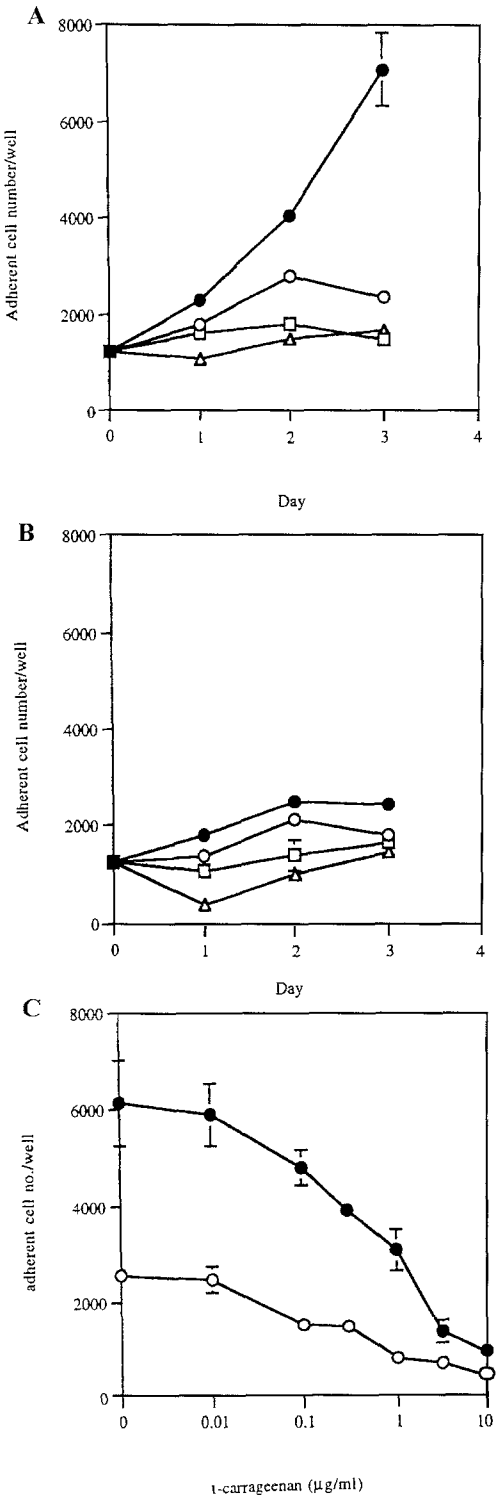


Fig. 2A–C Inhibition of FBHE proliferation by ι-carrageenan. Cells were plated at 1000 cells/well, left over-night and grown in medium **a** in the presence of bFGF or **b** in the absence of bFGF in medium containing no ι-carrageenan (●), 1 μg ι-carrageenan/ml (○), 3 μg ι-carrageenan/ml (□) or 10 μg ι-carrageenan/ml (△). **c** Dose-response curve of ι-carrageenan against FBHE cells grown in the presence (●) or absence (○) of bFGF. *Error bars* represent the SD of triplicate determinations and are smaller than the symbol when not shown.

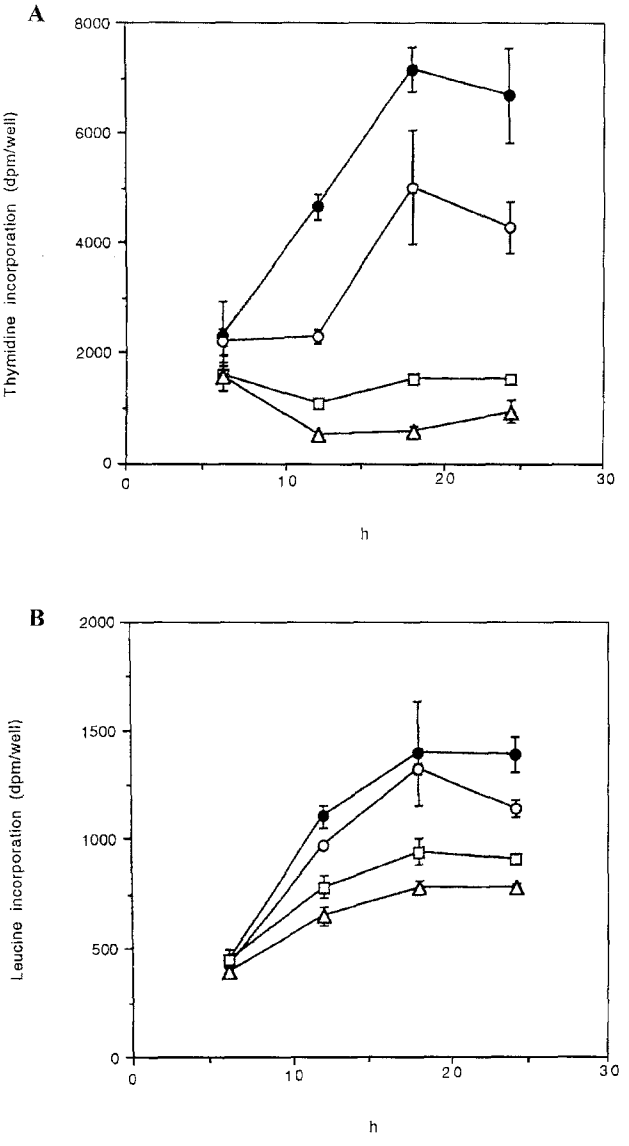


Fig. 3A, B Inhibition of **A** FBHE DNA synthesis and **B** FBHE protein synthesis by ι-carrageenan. Cells were grown in medium containing no ι-carrageenan (●), 1 μg ι-carrageenan/ml (○), 3 μg ι-carrageenan/ml (□) or 10 μg ι-carrageenan/ml (△). *Error bars* represent the SD of triplicate determinations and are smaller than the symbol when not shown

These doses of ι-carrageenan completely suppressed further DNA synthesis (Fig. 3a). By contrast, protein synthesis was not inhibited at 6 h, and protein synthesis continued to increase for 18 h after the addition of ι-carrageenan, although at levels lower than those observed in untreated cells (Fig. 3b). For all concentrations of ι-carrageenan examined, there was substantially greater inhibition of DNA synthesis than of protein synthesis. For example, at 18 h after the addition of 3 μg ι-carrageenan/ml, DNA synthesis represented 7% of control values whereas protein synthesis was 55% of control values. κ-Carrageenan was a significantly weaker inhibitor of DNA synthesis of FBHE cells than was ι-carrageenan, exhibiting an IC_{50} value of 170 μg/ml.

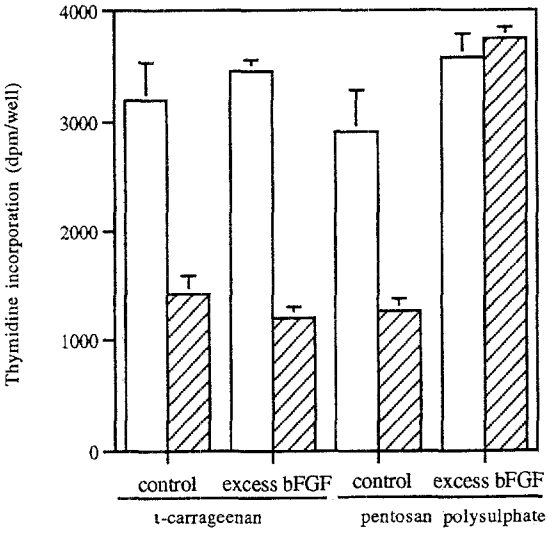


Fig. 4 Effect of excess bFGF on the inhibition of DNA synthesis of FBHE cells by ι-carrageenan or pentosan polysulphate. Untreated cells (*open columns*) or cells treated with 2 μg ι-carrageenan/ml or 20 μg pentosan polysulphate/ml (*hatched columns*) were grown in the presence of 10 or 1000 ng bFGF/ml and DNA synthesis was determined 24 h later. Data are means values ± SD for triplicate determinations.

Excess bFGF did not reverse the inhibition of FBHE DNA synthesis by ι-carrageenan (Fig. 4). This indicates that inhibition of bFGF binding is not the primary mechanism for the inhibitory effect of ι-carrageenan. By contrast, inhibition of DNA synthesis by pentosan polysulphate was completely prevented by the addition of excess bFGF (Fig. 4).

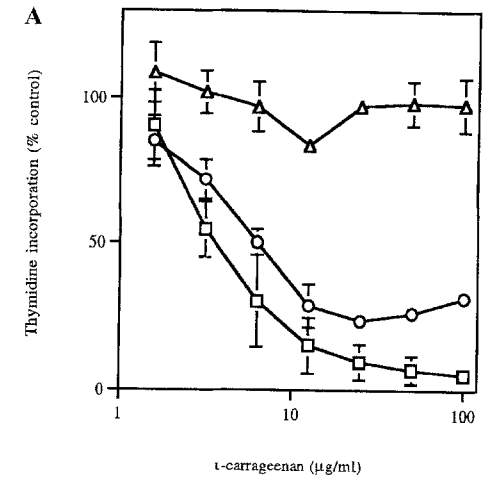
DNA synthesis of cell lines

Various cell lines in exponential growth were treated with a single administration of ι-carrageenan and DNA synthesis was determined 24 h later. Longer exposure periods did not significantly enhance the inhibitory effect (data not shown). The spectrum of effects is illustrated in Fig. 5a and summarised in Table 2. Cells can be categorised into three groups in terms of the level of inhibition of DNA synthesis induced by ι-carrageenan: those weakly inhibited (< 20% inhibition by 100 μg ι-carrageenan/ml), those partially inhibited (20%–80% inhibition by 100 μg ι-carrageenan/ml), and those inhibited to > 80% by 100 μg ι-carrageenan/ml (Table 2). FBHE and LNCaP were the cell lines most sensitive to inhibition of DNA synthesis by ι-carrageenan. The oestrogen-receptor-positive breast cancer lines MCF-7 and T47D, the oestrogen-receptor-negative cell line SKBR3 and the non-small-cell lung cancer (NSCLC) cell line L23/P and its multi-drug-resistant variant L23/R were partially inhibited. The rest of the lines examined were inhibited to < 20%. There was no correlation between the organ of origin of the cell line

Table 2 Effect of ι-carrageenan on the proliferation and DNA synthesis of various cell lines

Cell line	DNA synthesis		Cell proliferation
	IC_{50}(μg/ml)[a]	% Inhibition by 100 μg ι-carageenan/ml	IC_{50}(μg/ml)[a]
Lung carcinoma			
L23/P	6	68	10
L23/R	6	53	13
MOR	> 100	42	> 100
BEN	> 100	14	> 100
LUDLU1	> 100	10	> 100
Breast carcinoma			
MCF7	32 ± 4 (n = 3)	60	> 100
T47D	14 ± 3 (n = 7)	65	45
SKBR3	16	72	> 100
Glioma:			
U87MG	> 100	38	> 100
Prostate carcinoma:			
LNCaP	4.5 ± 1.7 (n = 10)	94	5.5 ± 1.9 (n = 6)
PC-3	> 100	17	> 100
Melanoma:			
A375	> 100	0	> 100
Non-tumourigenic lines:			
BEAS-2B	> 100	16	> 100
Swiss 3T3	88	52	> 100
FBHE	1.8	98	2.8

[a] Data are mean values ± SD or mean values for of 2 independent determinations when no SD is shown.



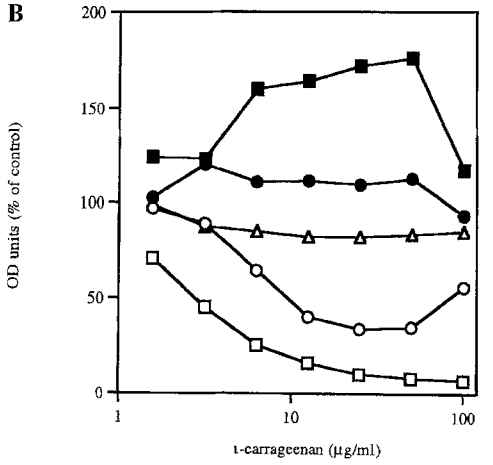
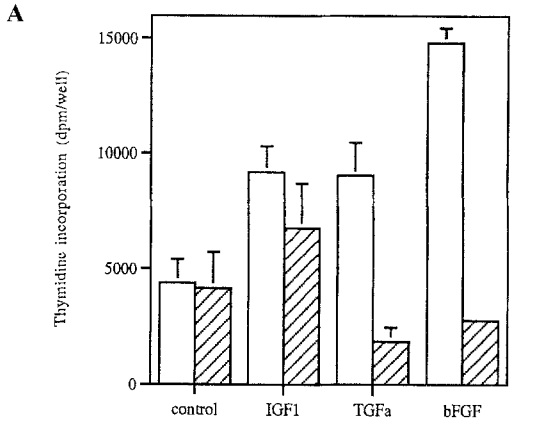


Fig. 5A–B Effect of ι-carrageenan on cell proliferation and DNA synthesis. **A** Effect on DNA synthesis of proliferating cells. Data are mean values ± SEM for n experiments on LNCaP (□ $n = 6$), PC-3 (△ $n = 3$) and L23/P (○, $n = 3$) cells. **B** Effect on proliferation as assessed by an MTT assay at 5 days after treatment with ι-carrageenan on LNCaP (□), L23/P (○), PC-3 (△), LUDLU1 (●) and BEAS-2B(■) cells. Data are mean values for 2 experiments.



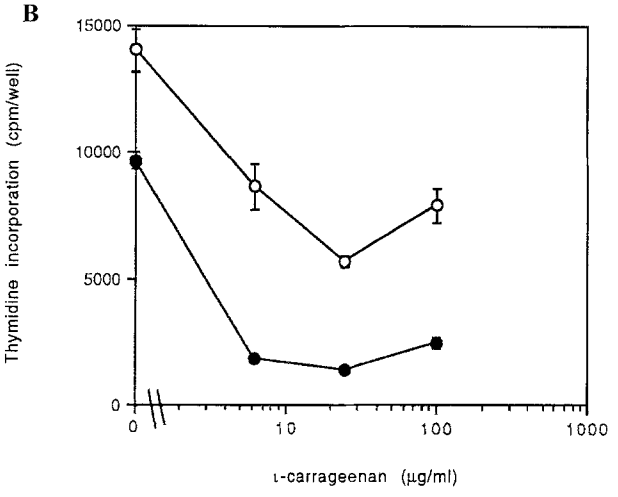


Fig. 6A, B Modulation of growth factor responsiveness of MCF-7 cells by ι-carrageenan. **A** MCF-7 cells growth-arrested in serum-free conditions were stimulated with growth factors (10 ng/ml) in the absence (*open columns*) or presence of 50 μg ι-carrageenan/ml (*hatched columns*) and DNA synthesis was determined after 24 h. **B** MCF-7 cells grown in 10% serum were treated with ι-carrageenan in the absence (○) or presence of 10 μg αIR3/ml (●) and DNA synthesis was determined 24 h later. Data are mean values ± SD and are smaller than the symbol when not shown.

and the sensitivity to inhibition. For example, although low concentrations of ι-carrageenan partially inhibited DNA synthesis of L23/P cells, two human squamous NSCLC cell lines BEN and LUDLU1 and the non-tumourigenic bronchial epithelial line BEAS-2B were only weakly inhibited. The most dramatic difference in sensitivity between two cell lines derived from the same organ was demonstrated by the two prostate carcinoma lines: DNA synthesis of the androgen-dependent prostate line LNCaP was inhibited to > 90% but that of the androgen-independent prostate line PC-3 was not significantly inhibited (Fig. 5a). Despite the potent inhibition of proliferating LNCaP cells by ι-carrageenan, basal DNA synthesis of quiescent LNCaP cells was not inhibited (data not shown). Heparin, which is structurally similar to ι-carrageenan, did not inhibit FBHE cells or the two prostate-carcinoma cell lines (IC_{50} values, > 100 μg/ml).

Inhibition of DNA synthesis by ι-carrageenan was further examined using MCF-7 cells growth-arrested in serum-free conditions. bFGF, TGFα and IGF-1 stimulated DNA synthesis of these cells (Fig. 6a). The cells did not respond to PDGF (B-chain homodimer; data not shown). Although basal DNA synthesis was not inhibited by ι-carrageenan, DNA synthesis stimulated by bFGF and TGFα could be completely inhibited (Fig. 6a), although in some experiments we found only partial inhibition of DNA synthesis by TGFα (data not

shown). By contrast, IGF-1-stimulated DNA synthesis either was not inhibited or there was only slight inhibition (Fig. 6a). Excess growth factor did not reverse the inhibition of DNA synthesis stimulated by bFGF or TGFα (data not shown).

Animal sera diluted in culture media contain sufficient IGF-1, and other members of the IGF family, to induce the proliferation of MCF-7 cells [24]. Consequently, we hypothesised that MCF-7 cells may escape total inhibition by ι-carrageenan when grown in serum by responding to IGF-like growth factors in the medium. This was examined using the anti-IGF-1 receptor antibody αIR3 to block the mitogenic activity of IGF-1 [25]. αIR3 alone caused only slight inhibition of DNA synthesis of MCF-7 cells in serum-containing medium (Fig. 6b). However, the partial inhibition of DNA synthesis of MCF-7 cells by ι-carrageenan was substantially enhanced in the presence of αIR3 (Fig. 6b).

Proliferation of cell lines

The anti-proliferative activity of ι-carrageenan was assessed by an MTT assay. Determinations were made 5 days after a single administration of ι-carrageenan for all cell lines except FBHE. Since FBHE cells are bFGF-dependent, ι-carrageenan was applied daily in fresh medium containing bFGF. The proliferation of FBHE cells and LNCaP cells was potently inhibited by ι-carrageenan (Fig. 5b, Table 1). These results are in agreement with the effects of ι-carrageenan on DNA synthesis. For a number of other cell lines, there was less inhibition of proliferation than of DNA synthesis (Fig. 5b, Table 1). Since the proliferation assay is carried out 5 days after treatment with ι-carrageenan, we investigated if inactivation of ι-carrageenan may be occurring during this period. However, daily administration did not enhance the anti-proliferative activity of ι-carrageenan against U87MG cells (selected since these cells are reported to require bFGF for proliferation [8, 29]; data not shown). The proliferation of BEAS-2B cells was enhanced by ι-carrageenan (Fig. 5b).

Cell-cycle analysis

A 24-h treatment with ι-carrageenan increased the proportion of LNCaP cells in the G1 phase and decreased the proportion of cells in the S phase in a dose-dependent manner (Fig. 7).

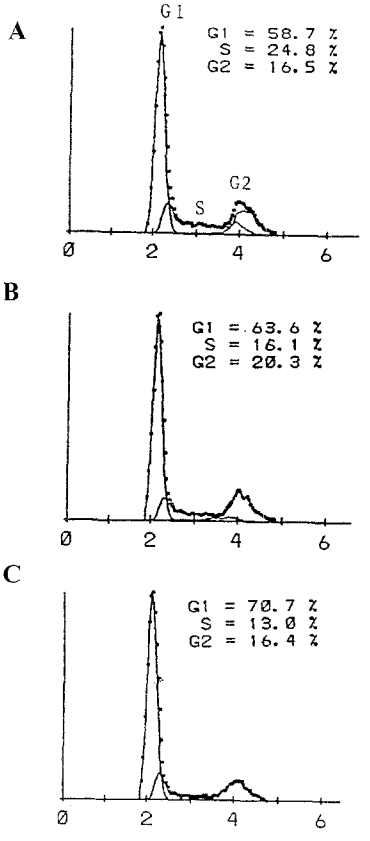


Fig. 7A–C Cell-cycle analysis of LNCaP cells. Cells were treated for 24 h with **A** no ι-carrageenan **B** 2 μg ι-carrageenan/ml, **C** 10 μg ι-carrageenan/ml

Discussion

This report demonstrates that ι-carrageenan potently inhibits DNA synthesis and proliferation of the endothelial cell line FBHE and the androgen-dependent prostate-carcinoma cell line LNCaP. Several lines of evidence indicate that ι-carrageenan is not a non-specific cell toxin: (1) anti-proliferative activity against a number of cell lines was either partial or absent; (2) ι-carrageenan inhibited DNA synthesis stimulated by bFGF and TGFα but not that stimulated by IGF-1; and (3) there was substantially less inhibition of protein synthesis than of DNA synthesis.

Cell-line selectivity is an important criterion for evaluating the potential of new agents as possible anticancer agents, and this criterion is used by the National Cancer Institute (NCI) for referring compounds for secondary testing [26]. In this study we used a panel of established cell lines, and further studies using primary cultures and tumour xenografts will be required for a full evaluation of the usefulness of ι-carrageenan for the treatment of androgen-dependent prostate cancer. The potent inhibition of the endothelial line FBHE is of particular interest. This line is derived from the aortic arches of bovine foetuses [27] and the ability of an agent to inhibit endothelial cells is an indication of possible anti-angiogenic activity. FBHE cells have proved useful in the evaluation of antagonists of tumour-derived stimulators of vasculature [18]. FBHE cells are, however, derived from macrovessel cell walls and

although some inhibitors of macrovessel cells also have anti-angiogenic activity [28], capillary formation during neo-vascularization requires the proliferation of microvessel endothelial cells. Consequently, we have recently confirmed that ι-carrageenan has anti-angiogenic activity in the chorio-allantoic membrane assay (unpublished observations).

One mechanism of action that probably contributes to the anti-proliferative action of ι-carrageenan is inhibition of binding of bFGF and, thus, inhibition of cells that require this growth factor for proliferation. In support of this hypothesis, inhibition of the proliferation of FBHE cells by ι-carrageenan correlates with the IC_{50} value reported for inhibition of bFGF binding (0.4 μg/ml) [6]. However, for κ-carrageenan there is a poor correlation between inhibition of bFGF binding (IC_{50} value, 7.7 μg/ml [6]) and inhibition of DNA synthesis (IC_{50} value, 170 μg/ml). bFGF is also implicated in the mitogenesis of several cell lines that were not inhibited by ι-carrageenan. For example, U87MG cells appear to require bFGF so as to sustain proliferation since both an antibody to bFGF and anti-sense to bFGF partially inhibit their proliferation [8, 29]. The lack of inhibition of U87MG cells by ι-carrageenan does not appear to be due to metabolic inactivation of this compound since there was no enhancement of the anti-proliferative activity with daily administration. bFGF is also an important mitogen for melanoma cells [30], but the melanoma line A375 was not inhibited by ι-carrageenan. One difference between FBHE cells and tumourigenic lines such as U87MG and A375 is that at low seeding concentrations, FBHE cells require an exogenous source of bFGF [27], whereas the tumourigenic lines do not. bFGF secreted by cells is retained at the cell surface in association with extracellular matrix [31]. Since ι-carrageenan only partially dissociates pre-bound bFGF [7], bFGF produced by U87MG and A375 cells may be retained at sites unavailable for inhibition by ι-carrageenan.

bFGF is implicated in the neo-vascularization of some tumours. Thus, a mouse tumour dependent on secretion of bFGF was inhibited to 70% by an anti-bFGF antibody. Since the antibody did not inhibit the tumour cells directly in vitro, inhibition of angiogenesis was implicated by the authors [32]. Also, inhibition of the bFGF-producing mouse tumour M5076 by suramin was countered by exogenous bFGF [33]. However, bFGF is only one of many angiogenic agents that have been identified. Recent reports have highlighted the importance of vascular endothelial growth factor (VEGF), a specific mitogen for capillary cells, for tumour neo-vascularization [34]. Many angiogenic molecules, including VEGF, bind heparin [16]. Since-carrageenan antagonises the binding of several heparin-binding growth factors, it is possible that this agent may also antagonise a number of heparin-binding angiogenic growth factors. The recent commercial availability of VEGF will allow us to test this hypothesis.

The inability of excess bFGF to reverse the inhibition of FBHE DNA synthesis by ι-carrageenan suggests that mechanisms of action other than bFGF antagonism may contribute to the anti-proliferative action. This conclusion is also re-inforced by our observation that ι-carrageenan inhibits TGFα-stimulated DNA synthesis of MCF-7 cells, despite the observation that ι-carrageenan does not inhibit TGFα binding [6]. We therefore addressed the possibility that ι-carrageenan is behaving as a heparin mimic. Both heparin and ι-carrageenan are polysaccharides with similar degrees of sulphation: heparin has two to three sulphates per disaccharide unit and ι-carrageenan has two sulphates per disaccharide unit. Heparin has anti-proliferative activity against some cell types and several targets for the anti-proliferative activity of this compound have been suggested, although the precise mechanism of action remains unresolved [20]. Heparin exerts a G1/S block [19] and, in this regard, ι-carrageenan is similar to heparin as it blocked LNCaP cells at the G1/S boundary. However, heparin and ι-carrageenan differ in several respects. Firstly, heparin did not inhibit either FBHE or LNCaP cells. In addition, the anti-proliferative action of heparin is reversed by EGF [35], whereas we have been unable to reverse the effects of ι-carrageenan with EGF (unpublished observations). Thus, we conclude that ι-carrageenan is not behaving simply as a heparin mimic.

The dose-response curves (to ι-carrageenan) of a number of cell lines grown in serum-containing medium were initially steep (low IC_{50} value) but then plateaued off (see Table 1, Fig. 5). We examined this phenomenon using MCF-7 cells, which can respond mitogenically to a number of growth factors, including bFGF, TGFα and IGF-1 ([24], present results). The IGF-1 receptor antibody αIR3 significantly enhanced the inhibition of DNA synthesis of MCF-7 cells by ι-carrageenan. Since ι-carrageenan inhibited DNA synthesis stimulated by bFGF and TGFα but not that stimulated by IGF-1 in defined medium, MCF-7 cells may have escaped total inhibition by ι-carrageenan in serum-containing medium by responding to IGF-like molecules (including IGF-1 and insulin) that are known to be present at mitogenically active concentrations in serum [24].

The degree of sulphation (i.e. sulphate groups per disaccharide unit) is an important determinant of the biological activity of heparin [36], and κ-, ι- and λ-carrageenans have degrees of sulphation of 1, 2 and 3, respectively. κ-Carrageenan had only weak anti-proliferative activity, whereas the more sulphated ι- and λ-carrageenans had higher anti-proliferative activities. However, the interaction of λ-carrageenan with serum proteins and the high anti-coagulant activity of this compound make it unfavourable for future development. Our observations suggest that for carrageenans, a degree of sulphation of 2 (i.e. ι-carrageenan) is optimal for anti-proliferative activity and minimal non-specific

interactions. An unfavourable property of ι-carrageenan, however, is its high molecular weight (250 kDa). Consequently, we are currently evaluating a number of low-molecular-weight derivatives. This study provides evidence that although antagonism of growth factors such as bFGF may contribute to the anti-proliferative activity of ι-carrageenan, this is insufficient to explain fully the anti-proliferative activity of this compound. Significant advances have recently been made in understanding the molecular interaction between heparin/heparan sulphate and bFGF, and this may ultimately result in highly specific bFGF antagonists [37, 38]. However, in the absence of a clear mechanism of action for ι-carrageenan, we currently favour a bioassay-driven approach to develop low-molecular-weight derivatives.

In summary, the present study demonstrates that ι-carrageenan potently and selectively inhibits the proliferation of LNCaP and FBHE cells at concentrations substantially lower than those that should affect blood coagulation. This compound has also been shown to have low toxicity in mice [5]. Thus, ι-carrageenan or derivatives of this compound are of interest for the treatment of androgen-dependent prostate carcinoma or as anti-angiogenic agents.

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