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Tumor-induced Growth-inhibitory Activity Isolated from the Yoshida Ascites Fluid by Extraction with Methanol*

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Abstract—*Growth-inhibitory activity was isolated from the Yoshida ascites fluid by sequential precipitation with polyethylene glycol, extraction with methanol, LH 20 Sephadex chromatography and preparative agarose gel electrophoresis. The cell non-specific activity was tumor-related as far as analogous fractions prepared from normal sera were inactive. Flow cytometric analysis indicates that the inhibition of cell growth was caused by blockage of the G_1–S and possibly the G_2–M phase transitions. The active component migrates electrophoretically associated to an unidentified α_1-antigen. In aqueous solution the molecular size of the inhibitor is ill defined, due to a tendency to autoaggregation and hydrophobic interaction with the chromatographic media. The molecular weight of the inhibitor as estimated by LH 20 Sephadex chromatography in methanol is approximately* 350 daltons. *This chromatographic fraction contains prostaglandin-E_2 cross-reactive material in amounts suggesting the participation of a prostaglandin derivative in the observed growth-inhibitory activity.*

INTRODUCTION

EXPERIMENTAL ascites tumors propagated in mice or rats normally kill the animals within 2 weeks. However, the tumor may be rejected or at least be subject to some inhibition of growth during the later stages of tumor development, probably due to the accumulation of so far undefined growth inhibitory humoral factors [1, 2]. For the Yoshida ascites hepatoma tumor-dependent humoral inhibitory activity was isolated with a fraction of the ascites fluid macroglobulins [3, 4]. However, an early humoral immunoresponse against the tumor was not responsible to a major extent for the observed growth inhibition [5]. Interferon can, as can related lymphokines, give rise to a similar non-selective inhibition of cell proliferation [6–8]. Host defence reactions often involve the interferon system; therefore the present investigation was carried out to evaluate the possible participation of this system in the growth-inhibitory effect associated to the fraction of Yoshida ascites fluid macroglobulins isolated by precipitation with 10% polyethyleneglycol [5].

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Abbreviations: FCS: fetal calf serum; PBS: phosphate-buffered saline, pH 7.4; PEG: polyethylene glycol 6000; PEG-fraction: macroglobulin fraction isolated by precipitation of cell-free ascites fluid or cell extracts with 10% PEG; AM-extract: ascites methanol extract prepared by extraction of the ascites fluid PEG-fraction with methanol; PGE_2: prostaglandin E_2; FCM: flow cytometry.

MATERIALS AND METHODS

Tumor cells

Yoshida hepatoma cells, obtained from the Fibiger Laboratory, Copenhagen, were propagated as ascites tumors in adult inbred female Wistar rats. JB-1 plasmocytoma cells propagated in AKR mice were obtained from the Institute for Cancer Research, Aarhus, Denmark. For *in vitro* culture the cells were propagated in RPMI medium containing 10% fetal calf serum (FCS). Viability of the tumor cells was determined by a dye exclusion test (trypan blue).

Ascites fluid

Ascites was collected on ice by peritoneal puncture on day 7 of tumor growth. Cell-free ascites fluid was isolated by centrifugation at 10,000 *g* for 15 min at 4°C. The samples were

processed separately or pooled and stored at -80°C.

Cell extracts

Tumor cells isolated after *in vivo* or *in vitro* culture were washed twice and resuspended in PBS to a density of 10^8 cells/ml, corresponding to the concentration of tumor cells in the ascites fluid. After lysis by repeated freezing and thawing, the cell extract was isolated as the supernatant collected after centrifugation at 20,000 *g* for 30 min.

Fractionation procedures

(1) Polyethylene glycol fractionation. Polyethylene glycol 6000 (PEG) was added in substance to the cell-free ascites fluid to a final concentration of 10% w/v. After incubation at 4°C for 20 hr the precipitate was isolated by centrifugation at 15,000 *g* for 30 min at 4°C. The sediment was washed twice by resuspension in PBS containing 10% PEG. For incubation experiments the washed precipitate was redissolved in the original ascites fluid volume of PBS.

(2) Methanol extraction. The washed ascites fluid PEG-precipitate prepared as described above was extracted by suspension for 1 hr at 20°C in the original ascites fluid volume of methanol. The ascites methanol extract (AM-extract) was separated from the remnance by centrifugation at 15,000 *g* for 30 min and evaporated to dryness.

For incubation experiments the AM-extract was redissolved in the original ascites fluid volume of PBS.

(3) Gel chromatography. The molecular size of the growth-inhibitory ascites fluid component was estimated by gel chromatography on Sephadex G 200 in PBS and Sephadex LH-20 in methanol. For the latter procedure a linear calibration curve was derived from the elution pattern of uridine (mol. wt, 244), prostaglandin E_2 (mol. wt, 252), prostaglandin $F_{2\alpha}$ (mol. wt, 255), vitamin B_{12} (mol. wt, 1355) and bacitracin (mol. wt, 1411). The chromatographic fractions were monitored by u.v. photometry at 280 nm.

The same fractionation procedures were used for the analysis of normal rat serum, FCS and cell extracts.

Preparative agarose gel electrophoresis

The AM-extract dissolved in 0.02 M diemal buffer, pH 8.6, was subject to electrophoresis in a 1.5-mm thick 0.5% agarose gel from a rectangular sample gel placed across and 30 mm from the cathodic end of a 100 × 200-mm glass plate. After electrophoresis at 6–8 V/cm for 1.5–2 hr the gel was cut in 20-mm wide sections in parallel with the sample gel (Fig. 4). The AM-extract antigens were separated from the gel by centrifugation at 15,000 *g* for 30 min. For incubation experiments the supernatants were dialyzed for 1 hr against PBS.

Immunoelectrophoresis

Immunoelectrophoretic analysis of the various ascites and serum methanol extracts was carried out as previously described [9] using antisera raised in rabbits by immunization with the Yoshida AM-extract.

Tumor cell proliferation

(1) [^{3}H]-Thymidine ([^{3}H]-thd) incorporation. The DNA-synthesis of exponentially growing tumor cells was estimated from their uptake of [^{3}H]-thd *in vitro*. Incubation was carried out in triplicate in microtest plates (NUNC, Roskilde, Denmark). To 4×10^4 tumor cells suspended in 100 μl RPMI medium containing 1% FCS was added 90 μl test sample and 10 μl of PBS containing 1 μCi of [^{3}H]-thymidine. The plate was incubated at 37°C in 5% CO^2 for 18 hr. Cells were collected by means of a Skatron cell harvester and radioactivity counted in a Packard Tricarb Liquid Scintillation Spectrometer (B2450) [4, 5].

(2) Flow cytometry. The cell cycle distribution of the tumor cells, incubated as above without thymidine, was estimated by flow cytometry as previously described [4]. The cells were lysed with Nonidet P40, and the nuclei stained with ethidium bromide and treated with RNAse. The measurements were done on a BioPhysics Cytofluorograph 4802A (Ortho Instruments, Westwood, U.S.A.). The DNA histograms were evaluated by means of a computer program for cell cycle analysis [10].

(3) Cell counts. The effect of ascites fluid and subfractions on cell proliferation was estimated from cell counts done simultaneously with viability tests and [^{3}H]-thymidine incorporation experiments.

Radioimmunoassay for prostaglandin E_2

The content of PGE_2 in the AM-extract was determined by radioimmunoassay using the PGE_2 RIA-kit of Boehringer, Mannheim, F.R.G. This assay works with a PGE_2 detection limit of 0.05 pmol and a cross-reactivity of 3.2% with PGE_1 and 0.2% with PGA_2, while the reactivity with other PG derivations was indicated as less than 0.05%.

PGE_2 Sigma cat. No. P.5640 was used as reference here as well as in the incubation experiments.

RESULTS

Yoshida ascites fluid and the isolated subfractions induced a dose-dependent inhibition of

tumor cell proliferation as estimated from cell counts and [^{3}H]-thymidine incorporation experiments (Table 1, Fig. 1). The inhibition of [^{3}H]-thymidine uptake caused by the AM-extract was, in contrast to that of mitomycin C and PGE_2, inversely related to the concentration of serum proteins in the incubation medium (Table 2).

Yoshida AM-extracts exerted a stronger inhibition on the DNA-synthesis of the Yoshida hepatoma cells than on that of the JB-1 plasmocytoma cells. However, the same difference in sensitivity was observed on incubation of the tumor cells with the JB-1 AM-extract or with mitomycin C.

As shown from the FCM analysis, the AM-extract induced a reduction in the proportion of cells in the DNA-synthesis (S) phase (Fig. 2). The influence of the ascites fluid fractions on cell proliferation was not caused by cytotoxicity, as shown by the dye exclusion experiments (Table 1).

Methanol extracts prepared from tumor host serum were equally active as the corresponding ascites fluid extracts, while analogous preparations derived from normal rat serum or tumor cell extracts were inactive or weakly active. Thus the growth inhibition of tumor cell methanol extracts never exceeded 10% of the activity of AM-extracts prepared from the corresponding cell-free ascites fluid. No significant difference was noted between the activity of methanol extracts prepared from tumor cells cultured *in vivo* or *in vitro*.

Table 1 compares the growth-inhibitory activity of the Yoshida ascites and the respective subfractions obtained by the sequential purification steps used for isolation of the inhibitor. Expressed in terms of activity vs optical density, the procedure used resulted in a more than 10^3-fold purification of the inhibitor.

After one extraction of the ascites fluid PEG-fraction with methanol no further activity was gained by re-extraction with methanol, ethanol,

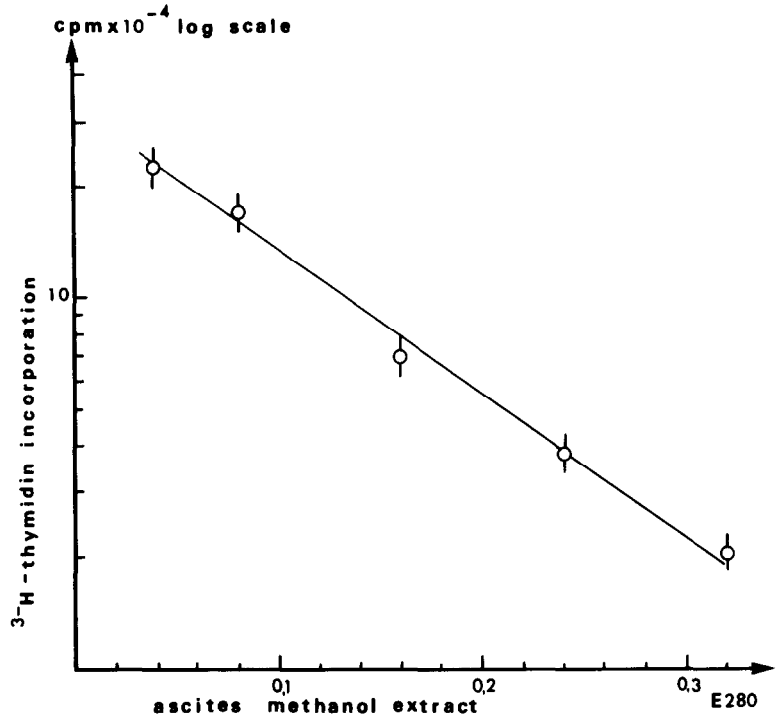

Fig. 1. Dose-dependent inhibition of tumor cell [^{3}H]*-thymidine incorporation induced by the Yoshida AM-extract (mean values ± S.E. of triplicate determinations).*

Table 1. Growth inhibition of Yoshida tumor cells incubated with ascites fluid and subfractions (mean values ± S.E.M. of 10 experiments)

Test material	% Inhibition [^{3}H]-Thymidine uptake	% Inhibition Cell proliferation	Viability (%)	Relative OD (280 nm)
1 Yoshida ascites fluid	9 ± 3	11 ± 3	82 ± 9	100
2 10% PEG fraction of 1	49 ± 7	53 ± 7	79 ± 9	20.9 ± 4
3 AM-extract of 2	81 ± 9	88 ± 9	69 ± 8	1.7 ± 0.2
4 Sephadex LH-20 peak fraction of 3	72 ± 8	79 ± 9	71 ± 9	0.7 ± 0.1

Table 2. The influence of serum proteins on the growth-inhibitory activity of the Yoshida AM-extract, prostaglandin E_2 and mitomycin C (mean values ± S.E.M. of 5 experiments)

Test material	% inhibition of [^{3}H]-thymidine incorporation in: PBS	FCS 1%	FCS 10%
Yoshida AM-extract (E280:0.21)	74 ± 8	32 ± 5	19 ± 4
Prostaglandin E_2 (5.0 μg/ml)	87 ± 9	83 ± 9	72 ± 8
Mitomycin C (0.4 μm/ml)	79 ± 9	81 ± 9	76 ± 8

ether or ethyl acetate. The molecular size of the inhibitory component in the Am-extract was approximately 350 daltons as defined by LH 20-Sephadex chromatography in methanol (Fig. 3). In aqueous solution the molecular size of the growth-inhibitory material of the AM-extract was ill defined due to a tendency to the formation of polymers and adhesion to the column materials. Electrophoretically the active component of the AM-extract migrated as an unidentified α^1-antigen (Fig. 4).

This antigen was absent from the analogous preparations derived from normal rat serum, while it was present in extracts of tumor cells grown *in vitro* or *in vivo*.

The growth-inhibitory activity of the AM-extracts was unaffected by proteolysis with trypsin at 37°C for 2 hr and resisted heating at 80°C for 5 min.

The prostaglandin radioimmunoassay demonstrated the presence of PGE_2 cross-reactive material in the AM-extract in amounts suggesting an involvement of a prostaglandin in the observed inhibition (Table 3).

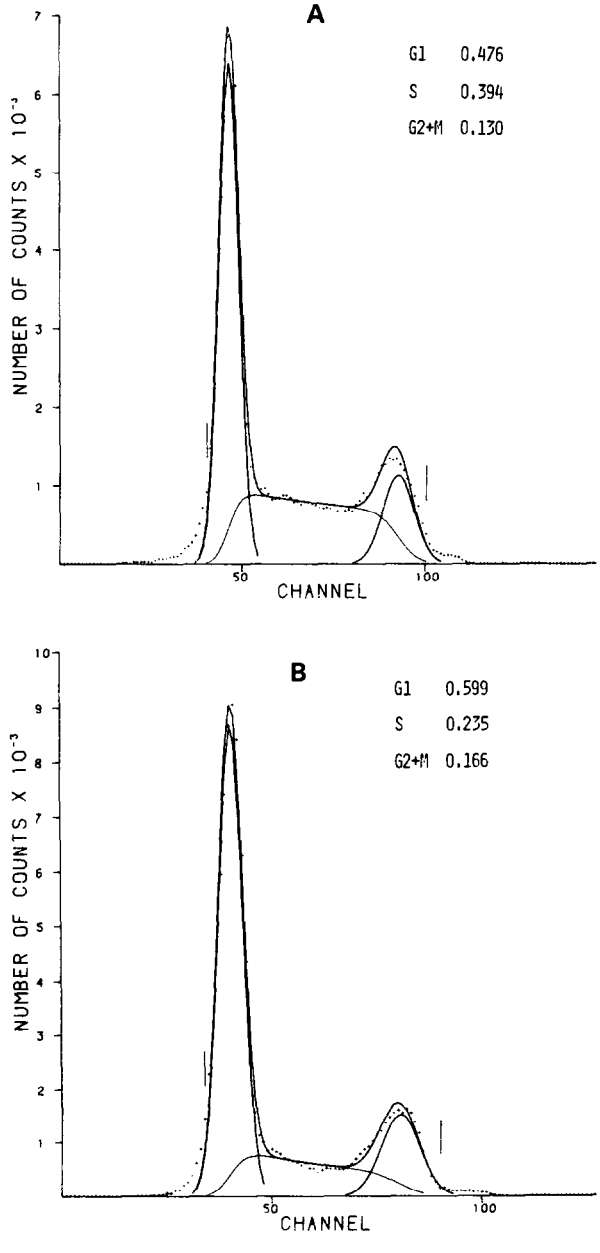

Fig. 2. Flow-cytometric analysis of tumor cell DNA distributions. (A) Control. (B) Tumor cells incubated with Yoshida AM-extract (E280: 0.21) causing an 81% inhibition of [^{3}H]-thymidine incorporation. Note in (B) the low proportion of cells in the DNA synthesis phase (S).

DISCUSSION

The present investigation demonstrates that the growth-inhibitory activity associated to the

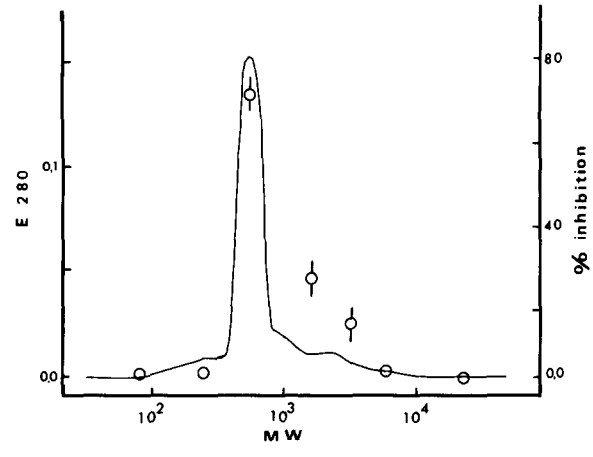

Fig. 3. Sephadex LH-20 chromatography of the Yoshida AM-extract. A 5-ml sample of the ascites methanol extract was loaded on a pharmacia column C16/70 and eluted with methanol at a flow rate of 12 ml/hr. Signatures indicate % inhibition of [^{3}H]-thymidine incorporation (O) and the optical density of the eluant at 280 nm (——). It appears that most of the growth-inhibitory activity is eluted as a single peak corresponding to a molecular weight of approximately 350 daltons.

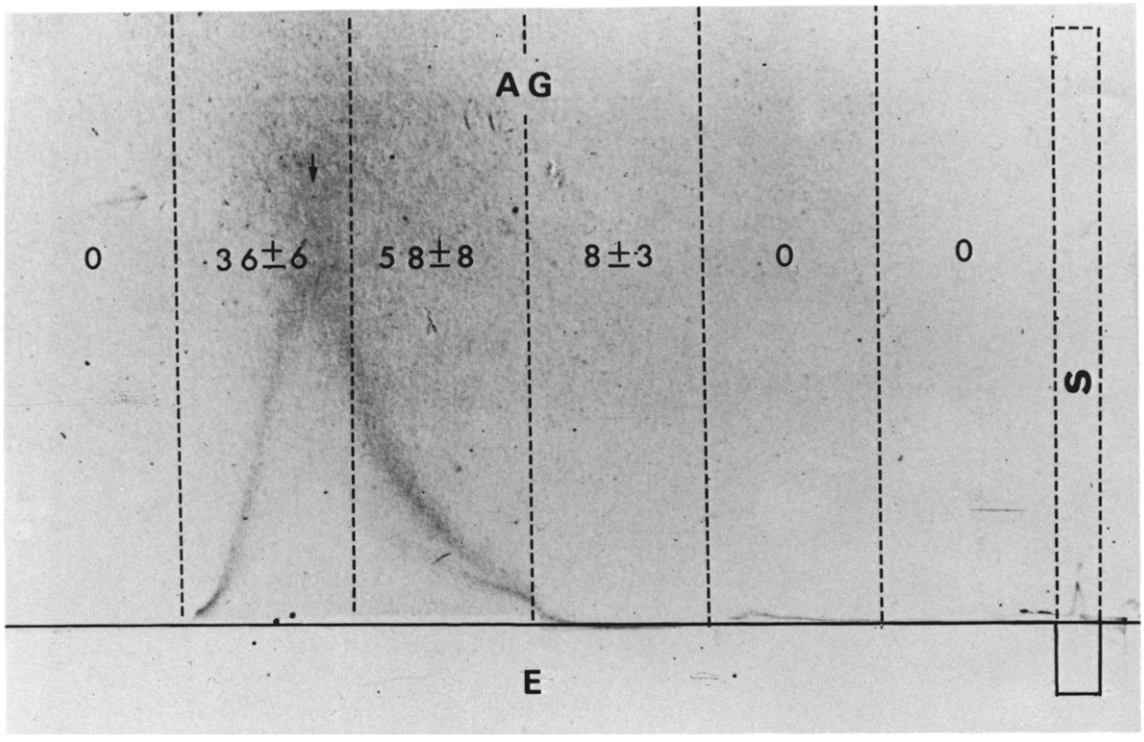

Fig. 4. Preparative and crossed immunoelectrophoresis of the Yoshida AM-extract. Preparative agarose gel electrophoresis: the sample gel S (1 × 5 × 80 mm) *contained* 600 µl *of Yoshida AM-extract concentrated by resolution in* 1/10 *of the original ascites fluid volume of electrophoresis buffer (Diemal, pH* 8.6, *I*:0.02). *Electrophoresis was carried out at* 7 V/cm *for* 1.5 hr. *Anode to the left. For incubation experiments the electrophoretic fractions were cut out in* 80 × 25 × 1.5-*mm gel selections (dashed lines) and processed as indicated in Materials and Methods. Numbers indicate % inhibition of tumor cell* [^{3}H]-*thymidine incorporation. Crossed immunoelectrophoresis: AM-extract antigens separated in the remaining part of the electrophoresis gel (E) were analyzed by electroimmunodiffusion in the* 1-*mm thick antiserum gel (AG) molded on the area of the plate left vacant after removal of the preparative electrophoresis gel sections. The antiserum gel contained* 10% *of rabbit antiserum against Yoshida AM-extract antigens. Electrophoresis was done at* 2 V/cm *for* 18 hr. *Anode at top. It appears that the growth-inhibitory activity migrates with the α-antigen (arrow) recognized by the AM-extract antiserum.*

Table 3. Prostaglandin E_2 content of the Yoshida AM-extract in relation to optical density (E210 nm) and growth-inhibitory activity (mean values ± S.E. of triplicate determinations)

Test material	PGE_2 (μg/ml)	E210 (nm)	% inhibition
AM-extract	2.6 ± 0.5	0.94 ± 0.3	74 ± 8
Prostaglandin E_2	5.0	0.23 ± 0.05	87 ± 9

Yoshida ascites fluid macroglobulins can be extracted with methanol. As indicated by FCM analysis of the cell cycle distribution, the inhibition induced by the AM-extract mainly concerned the G_1–S phase and possibly also the G_2–M phase transitions. In other cell systems a similar G_1 phase blockage was observed under influence of the interferons [8] and chalones [11]. Moreover, these substances resemble the present inhibitor in respect to solubility in organic solvents and tendency to hydrophobic interaction in aqueous solution [1, 8, 11]. Reversible protein-binding of the active component in the AM-extract could explain the neutralizing effect of serum. Specific carrier proteins have not been identified; however, preferential association of growth-inhibitory activity to a few ascites fluid proteins have been recognized [5]. The nature of the α-antigen in the AM-extract is uncertain, as is its possible participation in the observed growth inhibition. This antigen co-fractionates with the inhibitor during LH-20 Sephadex chromatography in methanol as well as by rechromatography and electrophoretic fractionation in aqueous solution, indicating a close association under these conditions. Molecular weight determination by gel filtration on LH-20 Sephadex may be biased by partition and absorption phenomena. However, in the present set-up, using methanol as an eluent, a linear relationship was found between elution volume and molecular weight for reference substances of highly different hydrophobicity. This indicates that at the defined conditions the elution pattern mainly resulted from a molecular sieving process. The small molecular size, heat stability and resistance against proteolysis argues against any relationship of the present inhibitor with the interferons or with the immunosuppressive β-globulin described by Cooperband *et al.* [12]. The activity of the AM-extract is tumor-related as far as analogous extracts of normal rat sera are inactive. The origin of the inhibitor has not been determined; however, the lack of activity of extracts prepared from lysates of tumor cells grown *in vitro* or *in vivo* suggests that the activity is generated by the host animal rather than from the tumor cells. This contrasts with the α-antigen, which apparently originates from the tumor cells.

The immunoassay demonstrates the presence of prostaglandin E_2 cross-reactive material in the AM-extract. However, the short half-life of PGE_2 *in vivo* [13] suggests that the immunoreactive material represents a conversion product (e.g. PGA_2) rather than the native PGE_2.

The lack of cell specificity in the growth inhibition induced by this preparation argues against any relationship with the chalones. However, the specificity of most of the chalones studied seems to depend on the involvement of high-molecular-weight carriers [1, 11]. Thus chalone effects might result from differences in cellular expression of specific receptor molecules or from cell-specific binding of mediator molecules carrying active prosthetic groups such as PGE_2.

Conflicting evidence has been presented of the possible significance of the prostaglandins in tumor development [14–16].

The concentration of PGE_2 required to inhibit cell proliferation *in vitro* [15, 17] is considerably higher than the concentrations observed in normal serum or tissue fluid [13]. However, PGE_2 is excreted in large amounts by stimulated mononuclear phagocytes [17], indicating that host defence reactions could give rise to a local build up of PGE_2 concentrations and thus leading to growth inhibition.

Evidence has been presented that PGE_2 participates in the antiviral and antiproliferative effects of interferon [7, 18]. In fact, a growth inhibitory prostaglandin species might represent the common principle searched for in the antiviral, growth-inhibitory and immunosuppressive activities of the various cytokines [6].

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