Europ. J. Cancer, Vol. 17, No. 4, pp. 421–431, 1981
Printed in Great Britain

0014-2964/81/040421-11 $02.00/0

Partial Purification and Characterization of a Cell Specific G_1-Inhibitor (Chalone) from JB-1 Ascites Tumors*

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Abstract—*Cell-specific G_1-inhibitory (chalone) activity has been extracted from old JB-1 ascites tumors and purified by means of G-15 Sephadex chromatography. Four peaks of activity were obtained, the amount of activity in each peak varying from batch to batch. The first eluting activity peak probably represents chalone activity present in an aggregated form. The latest eluting activity peak was totally unspecific and could be attributed to the presence of high amounts of salts. The two intermediary peaks of activity were investigated in more detail. It is shown that the first eluting activity peak of these two is due to the polyamine spermine complexed to a carrier. Although the spermine complex exhibits a certain degree of cell-specific inhibitory activity* in vitro, *it is totally inactive* in vivo. *The second eluting activity peak containing the main part of cell-specific G-inhibitory activity has been characterized as a small molecular weight* (M_r 300–600), *ampholytic, hydrophobic, slightly acidic, and thiol-containing peptide active both* in vitro *and* in vivo.

INTRODUCTION

IT IS well documented that the growth rate of experimental ascites tumors generally decreases with increasing tumor age and cell number [1–4]. This decrease is accompanied by an increasing accumulation of non-cycling tumor cells in resting G_1 and G_2 compartments [1]. In the cell-free ascites fluid from the JB-1 ascites tumor in the plateau phase of growth we have found substances with molecular weights less than 50,000 D which reversibly and specifically arrest JB-1 cells in G_1 and G_2 [5–8]. The effects of these substances are in several ways similar to that of normal cell-specific growth regulators termed chalones (for review articles, see [9, 10]). Since more than 80% of the non-cycling cells in plateau tumors are in G_1, and these cells are responsible for most of the recycling given the proper conditions, we have concentrated our efforts upon the purification of the G_1-inhibitor or G_1-chalone.

Accepted 28 October 1980.
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†Sponsored by the Danish Cancer Society.

MATERIALS AND METHODS

Tumors

The JB-1 ascites tumor [11] is a hypotetraploid transplantable plasmacytoma maintained syngeneically in inbred AKR/Aa mice aged 2–3 months by serial i.p. inoculation of 5×10^6 tumor cells every ninth or tenth day. The $L1A_2$ ascites tumor is a hypotetraploid tumor cell line developed from C3Hf mouse lung tissue [12] and maintained in the C3H/Aa strain of mice aged 2–3 months by weekly i.p. inoculations.

Preparation of G_1-inhibitory extracts

Procedure 1. Ascitic fluid obtained by aseptic aspiration of the JB-1 ascites tumor 11–13 days after i.p. inoculation of 5×10^6 tumor cells is centrifuged at 1500 ***g*** for 10 min at 0–4°C. Subsequently, the acellular ascitic fluid are ultrafiltered at 0–4°C through an Amicon membrane XM50 (Amicon B.V.) with a cut-off level of 50,000 D.

Procedure 2. Packed JB-1 cells obtained by centrifugation of the ascites as described in procedure 1 is resuspended in a volume of Hank's balanced salt solution (Grand Island Biological Co.) (supplemented with 0.01%

$NaHCO_3$) corresponding to the original volume of ascites (approximately 10^8 tumor cell/ml). The resuspended cells are gently stirred for 5 hr at 37°C in an atmosphere of 95% air and 5% CO_2. The pH of the incubation mixture is 7.3–7.4. Subsequently, the cells are centrifuged at 1500 ***g*** for 10 min. On practical purposes the supernatant is not routinely XM50 ultrafiltered since the content of high molecular weight material is less than 5% of that of cell-free ascites and can be removed in the subsequent G-15 Sephadex chromatography purification step.

The extracts from procedures 1 and 2 are stored at −80°C until fractionation on G-15 Sephadex.

In vitro G_1 *inhibitor assays*

The *in vitro* test system has been described elsewhere [13, 14]. In brief, JB-1 or $L1A_2$ cells (the latter cell line used for the estimation of cell specificity) established *in vitro* are synchronized by starvation. In starved cultures, 85% of the cells are in G_1, and when cells from these cultures are explanted to test cultures with fresh medium, a synchronous wave of cells will traverse the cell cycle. The G_1-inhibitory activity is estimated as a delay of the wave of synchronous cells travelling from G_1 into the S phase.

The percentages of cells in S phase are estimated from the DNA histograms obtained by flow cytometry as recently described [13]. The inhibitory activity or the percentage of inhibition has been calculated from

$$\% \text{ inhibition} = \frac{(\% \text{ of cells in S phase in control} - \% \text{ of cells in S phase in sample})}{(\% \text{ of cells in S phase in control})} \times 100.$$

In vivo *test system*

Principally, the *in vivo* test system is very similar to the *in vitro* test system described above [13, 14]. In plateau phase of growth, approximately 45–60% of JB-1 and $L1A_2$ tumor cells are in G_1 phase. After retransplantation of plateau phase tumor cells to new hosts an entry of G_1 tumor cells into the S phase is observed. The G-inhibitory activity is estimated as a delay of the wave of synchronous cells travelling from G_1 into the S phase [15].

G-15 Sephadex chromatography

Freeze-dried extracts are fractionated on a G-15 Sephadex (Pharmacia Fine Chemicals) column (5.0 × 90 cm) for preparative purposes with 0.05 M CH_3COOH at a flow rate of 110 ml/hr at 4°C. Fractions of 10 ml are collected in a fraction collector. The void volume (V_0 = 475 ml) has been determined with Blue dextran (Pharmacia Fine Chemicals). The total bed volume V_t has been found to be 3.05 × V_0. The absorbance at 254 nm of the eluate has been measured continuously with an LKB 2089 Uvicord 3 (LKB) and printed out on a chart recorder. To measure the radioactive substances eluted from the columns aliquots of 50 µl from each fraction have been placed in scintillation vials with the addition of 10 ml of INSTA-GEL (Packard Instrument Company). The vials are counted in a Beckman liquid scintillation counter (Beckman Instruments International S.A.). Biological activity has been estimated on 50–200 µl of every fifth fraction after neutralization with equal volumes of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid) (Sigma) in 0.3 M NaCl, pH 7.6.

Molecular weight of G_1-inhibitory activity was estimated using the following known marker substances (molecular weight and estimated K_{av} in parentheses) run separately on the same column: (1) Bacitracin (1450) (0.1) (Schwarz/Mann); (2) oxidized glutathione (614) (0.22) (Fluka); (3) mixed disulfide of cysteine and glutathione (410) (0.44); (4) spermine–phosphoethanolamine complex (343) (0.30); (5) glutathione (307) (0.50) (Fluka); (6) Met-Ala-Ser-tripeptide (306) (0.34) (Becton-Dickenson); (7) γ-Glu-Glu dipeptide (276) (0.52) (Serva); (8) spermine (202) (0.72) (Sigma); (9) methionine (149) (0.63) (Merck) and (10) leucine (131) (0.67) (Merck). Marker 3 was prepared from cystine (Sigma) and glutathione according to Eriksson and Eriksson [16]. Marker 4 was prepared by incubating 20 mg spermine tetrachloride and 20 mg phosphoethanolamine in 3 ml of water for 2 hr at 21°C; the complex was isolated from non-complexing spermine and phosphoethanolamine by G-15 Sephadex chromatography. The markers were detected with ninhydrin reagent.

In the tracer exchange experiments a smaller G-15 Sephadex column (2.5 × 40 cm) has been used.

Conductivity analysis

The conductivity in the column eluates is measured continuously with a Philips PW

9513 flow electrode (Philips A/S), and an LKB 5300 B conductolyzer (LKB), and printed out on a chart recorder. Specific conductance has been calculated from

$$S.C. = \frac{K}{Rm},$$

where $S.C.$ = specific conductance in mho/cm, K = cell constant in cm^{-1} and Rm = measured cell resistance in ohm.

Ion exchange experiments

CM-Sephadex C-25 cation exchange chromatography. Amounts of 30–50 ml of extract or G-15 Sephadex fraction (the latter adjusted to pH 7.4) with equal volumes of 50 mM HEPES in 0.3 M NaCl are passed through a column (0.7 × 4.0 cm) packed with 250 mg of CM-Sephadex C-25 (Pharmacia) preequilibrated with 50 ml of 0.15 M NaCl containing 50 mM HEPES pH 7.4 at a flow rate of 20 ml/hr. For G-15 Sephadex chromatography analysis the eluate is concentrated 10 times by freeze-drying.

Dowex chromatography. One gram of each of Dowex AG1-X2 (anion exchanger) and Dowex AG50 W-X2 cation exchanger (Bio-Rad laboratories) are washed with 10 ml of 1 N NaOH, 10 ml of H_2O and 10 ml of 1.0 M CH_3COOH pH 2.5 or 10 ml of 1.0 M CH_3COONa buffer pH 6.0. The ion exchanger is poured into small columns of 0.3 × 3.0 cm and equilibrated by elution with 20 ml of 0.05 M CH_3COOH pH 3.0 or 0.05 M CH_3COONa pH 6.0. Freeze-dried peak 3 dissolved in 2.0 ml of 0.05 M CH_3COOH or CH_3COONa has been passed through the columns. The eluates have been freeze-dried and tested on JB-1 cells *in vitro.*

Proteinase K treatment

A sample of peak 3 is dissolved in 500 µl of 0.15 M NaCl + 0.05 HEPES pH 7.4 containing 25 µg proteinase K (Merck) and incubated for 1 hr at 35°C and subsequently tested *in vitro* on JB-1 and $L1A_2$ cells.

High voltage paper electrophoresis

A sample of peak 3 has been spotted on Whatman 3 MM paper (Reeve Angel & Co. Ltd.) and exposed for 30 V cm^{-1} for 60 min. A mixture of amino acids, γ-Glu-Glu dipeptide (Serva) and a mixed disulfide of glutathione and cysteine (the latter prepared according to Eriksson and Eriksson [16]) used as standards are run in parallel on the same paper. A guide strip containing 1/10 of the applied sample + standards has been stained with cadmium–ninhydrin reagent. The other 9/10 of the paper has been cut into pieces and eluted with 100 mM NH_3 in H_2O, freeze-dried and tested on JB-1 and $L1A_2$ cells in culture.

Treatment with iodoacetamide

A sample of peak 3 is dissolved in 1.0 ml of 0.16 M sodium phosphate pH 8 containing 0.3 M 2-mercaptoethanol (Calbiochem). After 1 hr at 25°C 500 µmol of iodoacetamide (Serva) is dissolved in 1.0 ml 1.0 M sodium phosphate pH 8 and incubated for 1 hr at 25°C. Finally, 500 µmol of 2-mercaptoethanol is added and the mixture fractionated on G-15 Sephadex.

Mercury agarose chromatography

The chromatographical material has been prepared according to the method described by Reeve *et al.* [17]. A mercury agarose column (0.2 × 1.0 cm) is successively washed with 2 ml of 10 mM $HgCl_2$ + 20 mM EDTA in 50 mM sodium acetate pH 4.8 and 200 M NaCl + 1 mM EDTA in a 100 mM sodium phosphate pH 6.0. The columns are equilibrated with 100 mM NH_4HCO_3 pH 8.0. Two milligrams of peak 3 is dissolved in 2.2 ml of NH_4HCO_3 buffer and passed through the column at a flow rate of 5 ml/hr. Then the column is washed with 2.0 ml of NH_4HCO_3 buffer and finally eluted with 2.0 ml of 100 mM NH_4HCO_3 buffer + 10 mM dithioerythreitol (Kleland's reagent) (Sigma) at a flow rate of 1.2 ml/hr. Fractions of 0.5 ml are collected, freeze-dried and tested on JB-1 cells *in vitro.*

RESULTS

Preparation of G_1-inhibitory extracts

Previous investigations [5] have shown that the highest amount of G_1-inhibitory activity in cell-free ascites of the JB-1 tumor is obtained after 2–3 days of plateau-phase growth corresponding to 11–13 days after inoculation. Fifty to seventy-five per cent of the total activity is consistently found in an XM50 ultrafiltrate of cell-free ascites [8]. The G_1-inhibitory activity may also be isolated from JB-1 tumor cells extracted with isotonic salt solutions.

The production of cell-specific G_1-inhibitory activity from JB-1 cells suspended in Hank's balanced salt solution reaches a maximum at 5 hr of incubation. Incubation for longer periods results in increased genera-

tion of unspecific inhibitors (results not shown).

Further ultrafiltration experiments indicated a molecular weight range of the inhibitory activity of less than 10,000 D (results not shown).

G-15 Sephadex chromatography of G_1-chalone activity

Owing to the apparent low molecular weight of the inhibitor(s) G-15 Sephadex was used. Figure 1 represents a typical elution from the G-15 Sephadex column. Four peaks of activity

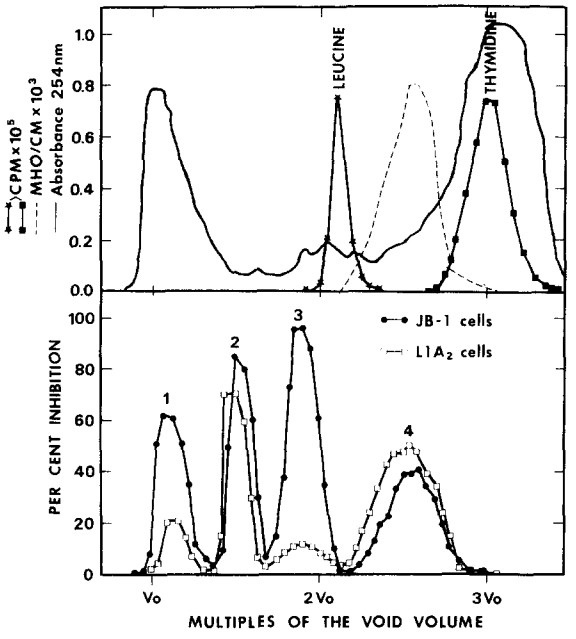

Fig. 1. Preparative G-15 Sephadex chromatography of G_1-inhibitory extract. Hank's balanced salt solution, 200 ml, conditioned for 5 hr with JB-1 cells, was freeze-dried and redissolved in 20 ml of 0.05 M CH_3COOH and applied to the column. Upper part: (1) absorbance at 254 nm, (2) specific conductance in mho/cm, (3) elution of 25 μCi [^{14}C]-methionine (first peak) and 50 μCi [^{3}H]-thymidine (second peak) cochromatographed with the extracts. In spite of a higher molecular weight of thymidine than methionine, thymidine is more retarded on the Sephadex column due to the presence of an extended aromatic π-electron system. (These internal markers have been used for the standardization of the column.) Lower part: Inhibitory effect on JB-1 and $L1A_2$ cells expressed as percentage of inhibition in comparison with respective controls.

are noticed. The molecular weights of the peaks have been estimated from the elution of various known markers determined in separate runs (see Materials and Methods). A molecular weight is found for peak 1, > 1500 D; peak 2, 500 D; peak 3, 300 D; peak 4 < 100 D.

Peak 1 corresponding to $K_{av} = 0$ and eluting in the void volume of the column was assumed to consist of aggregates of different inhibitors with higher molecular weight factors since this peak was always absent in starting material previously purified by means of XM50 ultrafiltration. When present, peak 1 represented 10–30% of the total inhibitory activity. Owing to the apparent inhomogeneity of this material it was not further studied.

The activity of peak 4 corresponding to $K_{av} = 1.1$ was assumed to be due to high concentration of salts since the conductivity peak coeluted with the inhibitory activity peak. No inhibitors other than the salts were present in this region of the eluate from the column. This was concluded from comparisons of the inhibitory activity of samples of peak 4 and Hank's balanced salt solution containing the same salt concentrations as judged from conductivity measurements. No additional contributions of inhibitory activity were found in peak 4 as compared with Hank's balanced solution.

Thus, peaks 1 and 4 were not studied in detail. Peaks 2 and 3 corresponding to $K_{av} = 0.30$ and $K_{av} = 0.44$ were selected for further investigations. Out of 43 consecutive chromatographical runs a double peak (containing both peak 2 and peak 3) was obtained 19 times, each peak representing 20–80% of the total activity. A single peak (peak 2) representing more than 80% of the activity was obtained 8 times. A single peak corresponding to peak 3 (more than 80% of the activity) was obtained 16 times.

The degree of cell specificity of peak 2 and peak 3 from the experiment shown in Fig. 1 was estimated from dose–response curves as seen in Fig. 2. The results indicate a low degree of cell specificity of peak 2 whereas peak 3 gives higher degree of cell specificity.

The cell-specific activity of peak 2 (obtained in 19 + 8 = 27 experiments) and peak 3 (obtained in 19 + 16 = 35 experiments) was estimated on each batch processed and a gradually increasing transition from totally cell-unspecific activity to very cell-specific activity was observed in each peak. However, the degree of specificity is far more expressed in most of the experiments with peak 3 as compared to peak 2. It is seen from Table 2 that cell-specific activity is obtained more than three times as often in the position of peak 3 as in the position of peak 2.

Characterization by ion exchange chromatography

The peaks of activity eluting from a G-15 Sephadex column were analysed by means of tracer exchange reactions with ^{14}C-labelled

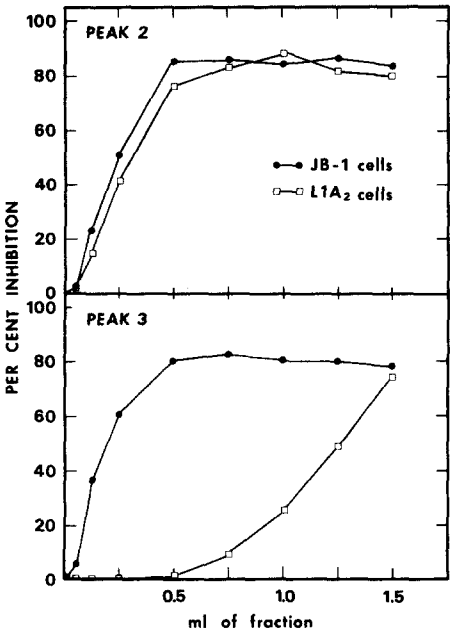

Fig. 2. Dose–response curves of peak 2 and peak 3 from the experiment shown in Fig. 1.

Table 1. Number of experiments with cell-specific and cell-unspecific activity in peak 2 and peak 3 from G-15 Sephadex column (Fig. 1)

	Peak 2	Peak 3
Cell specific*	7	24
Cell unspecific†	20	11

*Exhibiting a dose–response curve similar to Fig. 3, lower part.
†Exhibiting a dose–response curve similar to Fig. 3, upper part.

spermine and CM-Sephadex C25 cation exchange chromatography as shown in Fig. 3.

Previous experiments have shown that the main part of inhibitory activity is due to spermine non-covalently bound to a negative acidic carrier [18]. The main carrier of spermine is phosphoethanolamine although a mixed disulfide of glutathione and cysteine is able to bind a certain amount of spermine [18].

Figure 3 illustrates the role of spermine in the chromatographical behaviour of the inhibitory peaks on G-15 Sephadex. Figure 3(A) indicates that peak 2 and to a minor degree peak 1 are able to bind ^{14}C-labelled spermine in contrast to peak 3. After CM-Sephadex chromatography all spermine is held back on the ion exchangers accompanied by loss of activity in peak 2 (to a lesser degree also of activity in peak 1) whereas peak 3 is largely unaffected (Fig. 2B). After addition of unlabelled spermine + labelled spermine to the CM-Sephadex eluate peak 2 reappears accompanied by spermine binding (Fig. 3C). The inhibitory peak at the position of spermine is due to a cytotoxic effect of spermine. Treatment of the CM-Sephadex column with 1.0 M NaCl after passage of extract through the column results in the elution of an inhibitory peak on G-15 coeluting with spermine (Fig. 3D).

From these results and others previously published [18] it is evident that the main part of the activity at the position of peak 2 is closely related to that of spermine and probably consists of a complex between spermine and a carrier.

Since peak 2 in some experiments exhibited some degree of cell specificity, the activity of the G-15 peaks before and after CM-Sephadex chromatography was investigated both *in vitro* and *in vivo* to estimate the role of spermine. (The *in vivo* assay is probably a more valid test system than the *in vitro* assay [15, 19].) The results are shown in Table 2. It is seen that although the spermine complex (peak 2) may exhibit some degree of cell specificity *in vitro* (does not inhibit the $L1A_2$ cells), it is inactive *in vivo*. Consequently the CM-Sephadex treatment of peak 2 is inactive both *in vitro* and *in vivo*. On the other hand, peak 3 is cell-specific and active both *in vitro* and *in vivo* and is not affected by CM-Sephadex.

Since peak 2 probably has no physiological relevance as a proliferation inhibitor *in vivo*, it was finally excluded from further investigations and the interest was concentrated on peak 3. A further purification of peak 3 was tried by means of Dowex ion exchanger chromatography.

Using Dowex ion exchangers equilibrated with 0.05 M sodium acetate–acetic acid buffers more than 95% of the activities could be bound to Dowex AG50 W-X2 (cation exchangers) at pH 3.0 and 6.0. The activity could be bound to Dowex AG1-X2 (anion exchanger) at pH 6.0 but not at pH 3.0. However, the activity could not be quantitatively eluted from the columns after binding.

Enzymatical and electrophoretical characterization

In two experiments, treatment of peak 3 with proteinase K resulted in complete in-

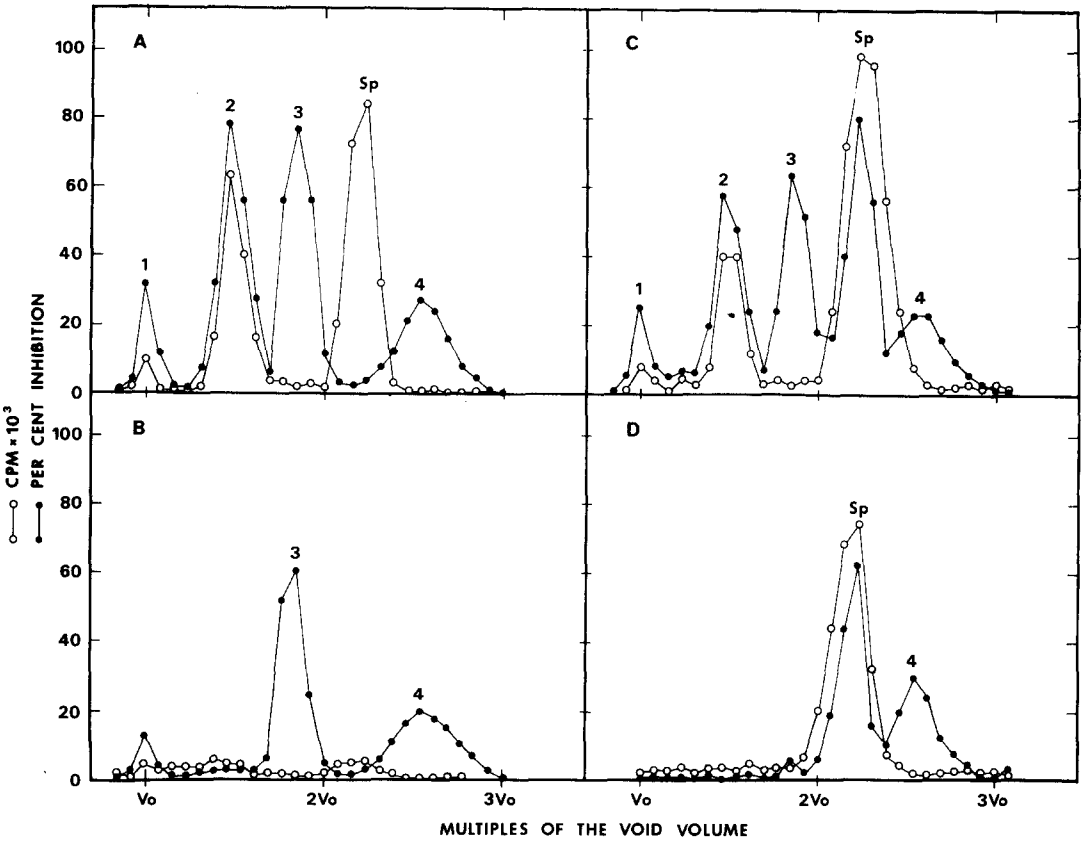

Fig. 3. G-15 *Sephadex chromatography analysis of* G_1*-inhibitory extract.* (A) *Fractionation of* 50 μCi ^{14}C-*spermine (Amersham) cochromatographed with the extract after* 2 hr *preincubation at* 21°C. (B) *Fractionation of CM Sephadex column eluate of* ^{14}C-*spermine + extract.* (C) *Fractionation of CM-Sephadex eluate after addition of* 50 μCi ^{14}C-*spermine* +2.0 mg *of spermine (Sigma).* (D) *After passage of* ^{14}C-*spermine + extract through the CM-Sephadex column, the bound material was eluted with* 1.0 M NaCl + 0.1 M HEPES pH 7.4 *and fractionated on G-15 Sephadex. Assay: JB-1 cells in culture.*

activation of the activity. In three experiments, the cell-specific activity was converted to totally unspecific activity. Proteinase K did not interfere with the assays when tested alone. Since the active compound might be a peptide, peak 3 was analysed by high voltage paper electrophoresis.

The electrophoresis analysis of peak 3 revealed at least 13 distinct ninhydrin positive spots (2 basic, 1 neutral and 10 acidic spots) as seen in Fig. 4. The cell specific activity was located in a yellow coloured spot (normal colour of the spot is pink to red) with an electrophoretical mobility a little slower than that of cysteine-glutathione.

Involvement of thiols in the chalone effect.

Figure 5 shows that, in contrast to $L1A_2$ cells, the proliferation of JB-1 cells is dependent to a certain degree on thiols present in the medium. A maximum effect on JB-1 cells is observed at 10^{-3} M mercaptoethanol. This dependence of thiols on the proliferation may be in connection with the chemical nature of the cell-specific inhibitor studied (see below).

Table 2. Percentage inhibition of peak 2 and peak 3

	In vitro				*In vivo**			
	Peak 2		Peak 3		Peak 2		Peak 3	
	JB-1	$L1A_2$	JB-1	$L1A_2$	JB-1	$L1A_2$	JB-1	$L1A_2$
Untreated	71	4	80	2	4	0	60	4
CM-Sephadex Eluate	−3	−5	72	0	0	−1	65	−2

*A three times higher dose was used *in vivo* than *in vitro*.

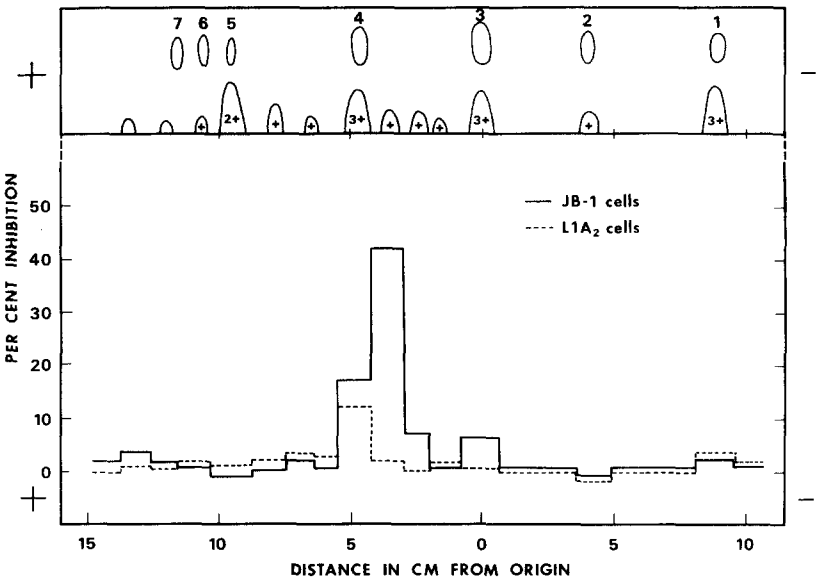

Fig. 4. High voltage paper electrophoresis of peak 3 from G-15 Sephadex column. For experimental conditions see Materials and Methods. Upper diagram shows the guide strip. The markers used are (1) Lys, (2) His, (3) Val, (4) Glutathione–cysteine mixed disulfide, (5) Glu, (6) Asp, (7) γ-Glu-Glu. The number of plus signs (+, 2+ or 3+) indicates semiquantitatively the ninhydrine colour intensity of the spots visible after electrophoresis of peak 3. Lower diagram shows inhibitory activity on JB-1 and $L1A_2$ *cells* in vitro *in eluate of the electrophoresis paper after cutting it into pieces.*

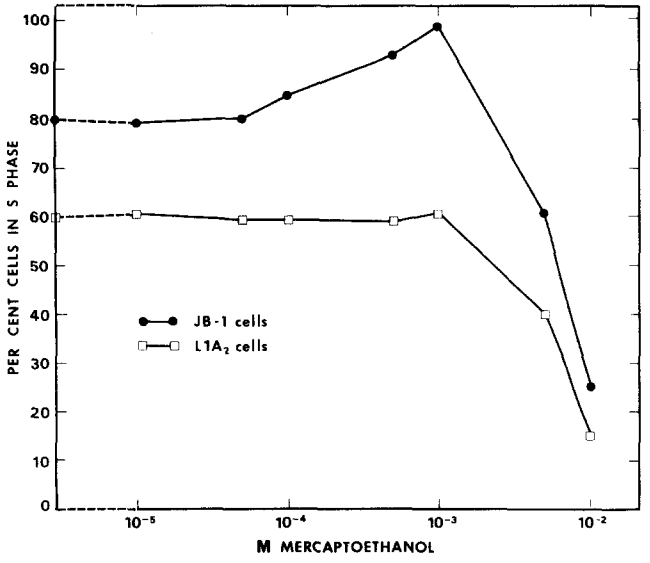

Fig. 5. Dose–response curves of 2-mercaptoethanol and JB-1 *and* $L1A_2$ *cells in cultures.*

Treatment of peak 3 with iodoacetamid after reduction with 2-mercaptoethanol did not give clear results since toxic products were generated which eluted from the G-15 column in the same way as untreated peak 3. Furthermore, attempts to test whether the inhibitory substance was labile to acid hydrolysis (6 N HCl at 110°C for 12 hr) were not successful since this treatment also caused the generation of toxic products in the chromatographical runs.

However, an indication of the involvement of thiols in the chalone effect observed was obtained by mercury agarose chromatography. As seen in Fig. 6, the activity is able to bind to mercury agarose and after saturation of the column with activity it can be eluted from the column with dithiothreitol. In con-

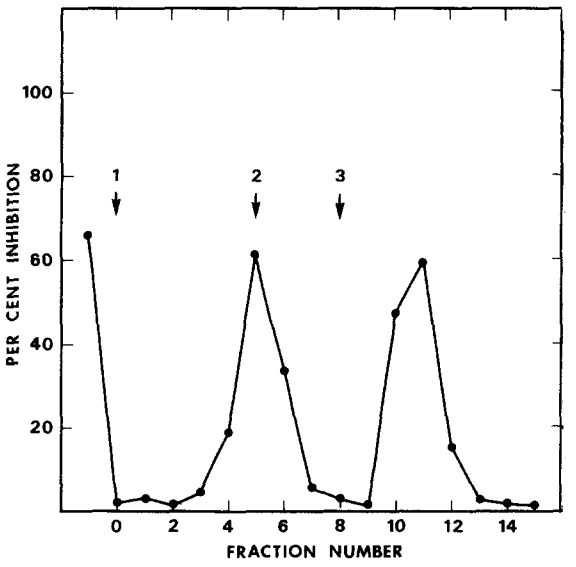

Fig. 6. Mercury agarose chromatography of peak 3 from G-15 Sephadex column. First point: Activity before application (66% inhibition). First arrow: Start of collecting eluate from the column. Second arrow: Outwash with buffer of excess of activity from the column due to saturation with activity. Third arrow: Elution with buffer containing dithioerythreitol.

trol experiments dithiothreitol by itself did not interfere with the assays.

DISCUSSION

The experiments described show that JB-1 ascites tumours in their plateau phase of growth produce specific and unspecific inhibitors of proliferation. These inhibitors may be studied separately after fractionation on G-15 Sephadex. The first peak eluted with the void volume probably represents an aggregate of different inhibitors with high molecular weight carriers and was excluded from further studies. All the inhibitory activity in the last (fourth) peak eluted is probably due to a cytotoxic effect of high salt concentration. The two intermediary peaks (peak 2 and peak 3) of activity from the G-15 columns were investigated in more detail. Although both peaks exhibited various degrees of cell specificity, the highest and most consistently found cell specificity could be attributed to peak 3.

Although there is great uncertainty in the estimation of molecular weights of low molecular weight substances by means of gel filtration, the two active factors of interest seem to have molecular weights between 300 and 1000 D. There is some discrepancy between the present data and the earlier data of Bichel [8], who found that the molecular weight of the G_1-chalone was 10,000–50,000 D based on ultrafiltration experiments. This discrepancy may be due to the uncertainty of the estimation of the low molecular substances, and to aggregation of the active factor(s) with other molecules, which often occurs during the processing of chalones (for a review see [10]).

The two intermediary activity peaks from the G-15 column may be comparable with lymphocytic chalone purification experiments by Ranney [20] who observed two discrete activity peaks eluting from G-10 Sephadex, both less than 1200 in molecular weight. The latest eluting peak contained the predominant activity and had an anionic character as judged from ion exchange chromatography [20]. Results very similar to those reported here have been described by Tanapat *et al.* [21]. Growth inhibitors produced by L1210 lymphoma cells were purified by G-10 Sephadex chromatography. Four peaks of activity were obtained, the first eluting in the void volume, the second as oxidized glutathione (molecular weight 612), the third as reduced glutathione (molecular weight 307) and the fourth eluting with the salts [16].

Characterization of the inhibitory peaks by means of tracer exchange reactions with ^{14}C-spermine and CM-Sephadex cation exchange chromatography indicates that more than 90% of the activity of the first peak (peak 2 in Fig. 1) is due to an effect of spermine. Previously published experiments suggest that spermine is non-covalently bound to primarily phosphoethanolamine, secondly to a mixed disulfide between glutathione and cysteine [5]. These two complexes have been synthetized chemically, e.g. by mixing spermine tetrachloride with phosphoethanolamine or cysteine–glutathione (the latter synthetized from cysteine and glutathione [3]) at low ion strength with subsequent purification on G-15 Sephadex. These two synthetic spermine complexes as well as the authentic isolated spermine complex have been tested *in vivo* and were inactive up to a dose of 100 mg/kg (results not shown).

Spermine alone or spermine in conjugation with carriers seems to be responsible for the activity of a number of "chalones" investigated [18, 22–25], but recent work indicates that these observations in some cases may be due to improperly purified preparations and in others to *in vitro* artifacts [26–29].

By means of CM-Sephadex chromatography the effect of spermine complexes could be eliminated and the main part of activity

effective *in vivo* could be attributed to the material eluting at the position of peak 3.

A further purification of peak 3 was performed using Dowex anion and cation exchangers. The activity was able to bind to both types of ion exchangers suggesting ampholytic character of the active molecule. However, quantitative elution from the columns was unsuccessful.

The inability for elution from the ion exchangers may be due to unspecific sticking to the ion exchanger resin owing to some hydrophobicity of the molecule studied. Thus, recent experiments indicate that the cell specific activity is soluble in CH_3OH, acidic acetone–water (80:20), but insoluble in acidic ethylacetate (i.e. the inhibitor is hydrophobic, but not of lipid nature).

Characterization by hydrolysis in 6 M HCl and blockade of a possible free thiol group in the molecule by means of carboxymethylation with iodoacetamid did not reveal conclusive results because of generation of cytotoxic products in the chromatographical runs. Possibly, more clear-cut results will be obtained with more purified preparations.

The treatment of the inhibitor with a proteolytic enzyme, proteinase K, suggests that the inhibitor may be a peptide. The conversions of the cell-specific activity in some of the experiments to unspecific activity may be due to generation of cytotoxic peptide fragments during the proteolysis.

Recent reports [27, 30, 31] suggest that the granulocytic, hepatic and lymphocytic chalone may be hydrophobic peptides since they can be conveniently purified by partition chromatography on a hydrophobic Sephadex matrice (LH-20 Sephadex) using 68% C_2H_5OH in H_2O.

The experiments with mercury agarose indicate that the inhibitor may contain a thiol in the molecule, probably present as cysteine. Purification by means of high voltage paper electrophoresis yielded a ninhydrin-positive compound which was analysed for amino acids after HCl hydrolysis. The analysis gave approximate molar ratios of 1 Asp, A Ser, 2 Glu and 2 Gly with traces of Thr and Ala. Thus, cysteine was not present in sufficient amounts to be detected. Explanations may be that (1) the active substance contains a thiol other than cysteine or (2) it may be very active and thus be present in low amounts, thus being totally hidden in the above-mentioned peptide. Thus, two or more peptides of similar electrophoretic mobility to the active substance may be present in the active spot. Although the activity is located in a ninhydrin-positive spot, there is still the possibility of the presence of a ninhydrin-negative inhibitory peptide with a blocked amino acid terminal. Normally the spot containing the active substance was obtained in low yield (10–20% of the activity spotted on the paper). However, most of the lost activity could be reactivated after electrophoresis by treatment with 10^{-3} M 2-mercaptoethanol.

Thiols seem to play a role in the effect of some low molecular weight endogenous growth inhibitors. Tanapat *et al.* [21] and Holmberg [32] have described thiol-containing peptides responsible for such activity. Recently, Paukovits [30] has isolated the granulocytic chalone which seems to be a low molecular weight peptide (molecular weight approx. 600) containing cysteine.

In conclusion, apart from artificial inhibitory factors, a JB-1 cell-specific inhibitor of proliferation has been partially purified and characterized. The substance may be a hydrophobic, acidic and thiol-containing peptide. Owing to the discrepancies between the characterization experiments and the amino acid analysis mentioned above, further purifications and biochemical characterizations will be necessary to elucidate the nature and also the biological function of the substance. Besides, other plasmacytomas and non-plasmacytomas will have to be tested in future work to further prove the criteria of cell specificity.

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