FRACTIONATION OF A CHALONE-CONTAINING PREPARATION FROM EHRLICH'S ASCITES TUMOR BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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In a previous study we attempted to fractionate active substances of a chalone-containing preparation (CCP) from Ehrlich's ascites tumor (EAT) of mice, by acting on the cells of this tumor in different phases of the mitotic cycle [1]. By gel-filtration of the CPP, isolated by alcoholic fractionation from EAT [3] on Ultrogel Ac-A-44 (LKB, Sweden), it was possible to separate the G_2-component of the CCP, inhibiting entry of the cells into mitosis, only partially from components inhibiting the entry of the cells into the phase of DNA synthesis (G_1 chalone) and the phase of DNA synthesis (S chalone) [2].

In this investigation, in order to achieve the same goal we used the method of gel-penetrating high-performance liquid chromatography (HPLC) by means of which, first, the original CCP can be more effectively separated, and second, the necessary number of CCP fractions can be quickly collected.

EXPERIMENTAL METHOD

The investigation was conducted on noninbred male albino mice of the same age (1.5-2 months), weighing 20-25 g. The animals were kept on a schedule of 12 h daylight alternating with 12 h darkness, and were fed ad libitum for 2 weeks before the experiment began. A diploid strain of EAT was used as the test object. CCP was obtained by alcoholic fractionation [3]. To separate the active components of CPP and to purify them further, the method of high-performance liquid chromatography (HPLC) was used. The CCP was dissolved in 50 mM phosphate buffer, pH 7.0. The solution of CCP (200 μl) in buffer with a protein concentration of 5 mg/ml was applied in each dilution to a TSG-GEL G2000 SW column (LKB, Sweden). The rate of elution was 0.5 ml/min. The protein concentration in the eluate was recorded at 226 nm in a continuous flow cuvette, by means of a Unicord instrument. The standard substances used to plot the calibration curve were blue dextran (2000 kD), ovalbumin (43 kD), chymotrypsinogen (25 kD), cytochrome c (12.5 kD) and potassium ferricyanide (0.3 kD). Fractions measuring 2 ml were collected in a test tube, in agreement with their elution profile (Fig. 1), and tested for biological activity, reflected in their action on the mitotic index – MI (the relative number of dividing cells), the radioactive index – RI (the relative number of DNA-synthesizing cells), and the intensity of labeling of the nuclei (ILN) with ^{3}H-thymidine in EAT cells on the 6th day of tumor development. MI and RI were expressed in promille. ILN was determined by counting the mean number of grains of silver above 50 nuclei. The total protein content in the samples was determined by the standard method of Lowry and co-workers [4]. The significance of differences between the values obtained was calculated by the Student–Fisher test. Differences were considered significant at the $p < 0.05$ level. Separation by this method was carried out four times: the results of one typical experiment are given.

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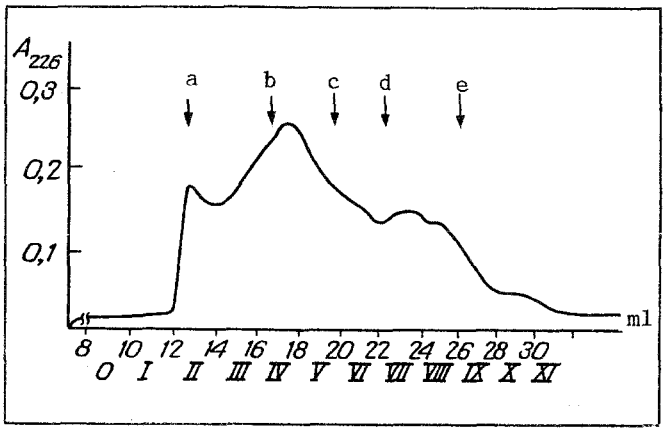

Fig. 1. Fractionation of chalone-containing preparation by HPLC. Abscissa, elution volume (in ml) and Nos. of fractions; ordinate, optical density at 226 nm. Arrows indicate times of injection of substances: a) blue dextran, b) ovalbumin, c) chymotrypsinogen, d) cytochrome, e) potassium ferricyanide.

TABLE 1. MI, RI, and ILN in EAT Cells 4 h after Injection of Various Fractions of CPP from EAT Obtained by HPLC (M ± m)

Fraction	MI	RI	ILN
K (control)	21,0±1,7	245,2±28,0	39,5±2,0
0	22,7±8,2	228,6±12,4	36,5±3,8
I	9,2±1,0* (39,1)	320,2±32,4	47,4±6,4
II	6,3±2,4**** (30,0)	292,5±11,5	37,4±2,5
III	7,4±2,1* (34,7)	194,4±20,0	34,6±2,4
IV	15,2±1,7**** (72,4)	258,2±30,9	27,8±5,8
V	3,0±0,5* (14,3)	243,8±8,8	39,7±5,5
VI	11,0±2,5** (52,4)	308,5±27,0	52,6±9,1
VII	17,5±1,8	109,8±22,5*** (44,7)	27,7±4,4**** (70,1)
VIII	16,8±3,5	200,8±36,8	35,8±5,3
IX	5,9±1,7* (28,1)	230,2±27,2	46,4±5,9
X	14,8±2,3**** (17,5)	254,5±24,5	70,6±14,3
XI	18,1±1,8	268,6±14,7	43,8±4,5
XII	16,8±3,4	307,6±42,4	50,2±9,0
Alcoholic precipitate (5 mg/ml)	8,1±1,1* (39,1)	211,6±22,6	40,5±3,5

Legend. *$p < 0.001$, **$p = 0.01$, ***$p < 0.01$, ****$p < 0.05$. Percentage of control value given in parentheses.

EXPERIMENTAL RESULTS

The profile of the fractionation curve of CCP from EAT by the HPLC method is illustrated in Fig. 1. Material of each fraction (from 0 to XII) was injected on average into five animals in a dose of 0.5 ml. Animals of the control group received an injection of 0.5 ml of buffer. The experiment began at 1 p.m. All the animals were given an injection of ^{3}H-thymidine (0.75 μCi/g body weight) 1 h before sacrifice (at 4 p.m.).

The results show that the component of CCP inhibiting entry of the cells into mitosis (G_2 chalone) was eluted in a broad front. It was present in all fractions except VII and VIII. These results correspond exactly to those obtained previously by gel-filtration on Ultrogel Ac-A-44 [1]. Meanwhile the G_1 chalone, inhibiting entry of the cells into the phase of DNA synthesis, and S chalone, inhibiting DNA synthesis directly, were present only in fraction VIII with molecular weight of about 12.5 kD. Thus by the method used it was possible to isolate from CCP a fraction containing a component affecting DNA synthesis in tumor cells, and evidently containing G_1 and S chalones.

The alcoholic precipitate of CCP, applied to the column, did not inhibit entry of EAT cells into the G_1 phase and did not directly inhibit DNA synthesis. It might be supposed that it did not contain G_1 and S chalones. The absence of an inhibitory effect of the alcoholic precipitate, in our view, can be explained on the grounds that, first, as the results of many experiments have shown, the time of onset of marked inhibition of the number of DNA-synthesizing cells is not strictly determined in time (it may be either before or 4 h after the beginning of the experiment) [2], and second, neutralization of the inhibiting (fraction VII) and stimulating (fractions I, VI, XII for RI and fraction X for ILN) action of components which, as Table 1 shows, are present in the alcoholic precipitate, evidently took place in the present experiment.

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