A NEW ENDOGENOUS INHIBITOR FOR MOUSE MELANOMA CELLS

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SUMMARY

The extract of B16 mouse melanoma was found to cause complete degeneration of the melanoma cells within 24~48 hours after the addition of the extract to the melanoma cell culture medium. This endogenous inhibitor was purified from the extract and characterized to be 4S cytoplasmic RNA.

Bullough and Laurence have reported the finding of "chalone," an endogenous antimitotic substance in epidermis (1) and melanoma (2). Chalone with epinephrine or with epinephrine and cortisone has been shown to suppress mitosis or cell growth not only in vitro but also in vivo (3). Mohr et al. (4) have shown that injections with chalone from pig epidermis caused the regression of Harding-Passey melanoma, a transplantable mouse melanoma.

We report here our finding of a new endogenous natural inhibitor of mouse melanoma. Unlike chalone, which is a protein and requires an additional hormone for its action, the newly isolated inhibitor is RNA, which does not need additional hormones for its action.

Both melanotic and amelanotic B16 mouse melanomas (5) were the source of the natural inhibitor. Two weeks after their transplantation

intramuscularly, the melanomas were harvested and stored at -20° C. Just before the preparation, they were thawed and homogenized with 3 volumes of redistilled water by the high-speed VirTis "45" Homogenizer (New York) for 3 to 5 min. The homogenate was centrifuged at 10,000 g for 10 min at 3° C and the supernatant was boiled for 14 min with frequent agitation for the first 4 min. Heavy coagulates were removed by centrifugation at 20,000 g for 30 min, and the clear supernatant fraction was condensed with Diaflo membrane (Amicon Corp., Lexington, Mass.) passed through Sephadex G200 with Tris-HCl buffer, pH 7.4, 5 mM. The elution pattern (Fig. 1) generally consisted of small and large peaks, the latter often showing a shoulder or minor

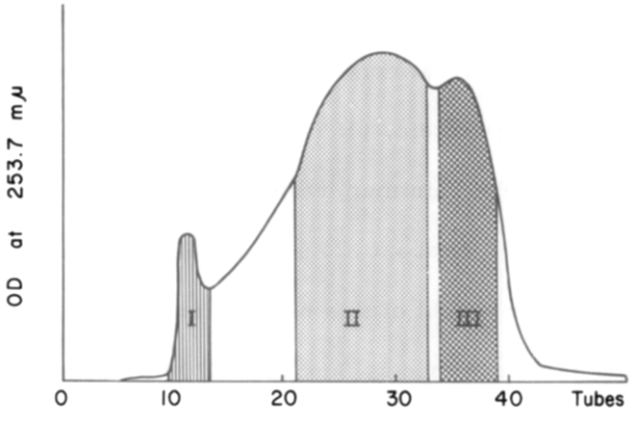

Fig. 1. Gel filtration of the boiled tumor extract.
Twenty thousand g supernatant fraction of the boiled tumor extract was passed through a Sephadex G-200 column 2.5 x 43 cm. It was eluted with Tris-HCl, 5 mM, pH 7.4 at a speed of about 20 ml/hr. Three representative fractions (I, II, and III) were subsequently concentrated and tested for their antitumor activity. The ordinate shows an arbitrary unit at 253.7 mμ (LKB UVICORD 4701A absorptiometer and 6510-A recorder) and the abscissa fraction tube numbers (6 ml each).

peak at the lower molecular weight side. We tentatively called these peaks fractions, I, II, and III. Peak I, which was eluted immediately after the void volume, is a protein fraction with the ratio of A260/A280 being less than one (0.5 ~ 0.8). Both Peaks II and III are RNA-rich fractions (A260/A280 = 2).

The concentration of protein and RNA was measured by the methods of Lowry et al. (6) and Dische (7) respectively. Each fraction was pooled, dialyzed, concentrated, and subsequently tested for inhibitory activity.

Monolayer cultures of B16 melanoma cells (Line HFH-18, subline P/51, see ref. 8) were used as an in vitro test system. Two hundred μl each of the test solution were sterilized by boiling for 10 min and added to the cell culture medium (3 ml). The results were read 24 to 48 hours after the addition of the test solution. The end point of this bioassay is cell death.

Only the fraction Peak II consistently caused complete degeneration of the melanoma cells. Neither trypsin nor nagarse digestion (10 $\sim$ 20 mg/mg protein, at pH 7.0 for 2 $\sim$ 3 hrs at 37° C) of this fraction decreased its inhibitory capacity. The treatment of this fraction with RNase, but not with DNase, (both 10 mg/mg protein, pH 7.0 for 3 hrs at 37° C) completely abolished its inhibitory action. The minimum effective dose of the purified extract (protein free Fraction II) is about .25 μ moles RNA/200 μl test solution / ml media. The molecular weight of this natural inhibitor ranges between about 20,000 $\sim$ 50,000 as determined by Sephadex G200 with known standards (Pharmacia, Uppsala, Sweden). Sucrose gradient centrifugation (9) of the fraction showed a single peak at 4 S (Fig. 2). Base analysis (10) yielded the nucleotide components of UMP 23.7, GMP 35.9, AMP 14.3, and CMP 26.1 moles per mole RNA respectively (n = 3), i.e. (G + C)/(A + U) ratio of 1.63.

Data thus far obtained clearly indicate that the new natural inhibitor isolated is a cytoplasmic RNA. The RNA extracts obtained in identical procedures from liver and brain of melanoma bearing mice did not show antitumor activity. We do not yet understand how it inhibits the melanoma cells, but it is certainly different from the action of chalone, which is a protein and

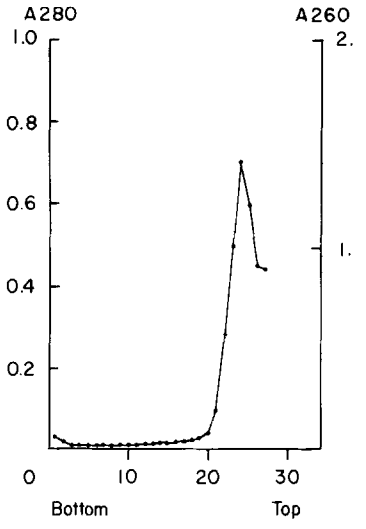

Fig. 2. Sedimentation of the RNA fraction (Peak II).
The RNA fraction having anti-melanocyte activity (100 μl) was layered on top of 5 to 20% linear sucrose gradient (4.9 ml) in Tris-HCl buffer, 5 mM, pH 7.4, and centrifuged in a SW 50 rotor at 3° C, in a Spinco L2 centrifuge at 50,000 rpm for 3 hours. After the centrifugation, 185 μl of each fraction was collected by puncturing the bottom of the tube and diluted with 1 ml of water, and the absorbance was read at 260 and 280 mμ. The peak corresponded to the standard tRNA, which was run simultaneously but in a different centrifuge tube. Note the absence of 28 S and 18 S cytoplasmic RNA.

requires the addition of epinephrine. A most attractive hypothesis at present is that the Peak II RNA fraction mediates cytotoxic immunity. Recent reports (11, 12, 13) describing the ability of RNA to transfer immunity may partially substantiate our assumption, though the production of cytotoxic immunity in melanoma remains to be proved.

Acknowledgment

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