A MACROMOLECULAR ANTITUMOR ANTIBIOTIC: MACRACIDMYCIN

Sir:

One of the authors, UMEZAWA,[1] has emphasized the importance of antitumor antibiotics with a macromolecular nature. In the continuation of the study of macromolecular antibiotics, a new peptide antitumor antibiotic named macracidmycin was isolated and characterized from the culture filtrate of *Streptomyces* sp. No. M590-G2 (ATCC 31104) which has been identified as *Streptomyces atrofaciens*.

Since the active substance in the culture filtrate had no antimicrobial activity, its production and purification were followed by determining activity against EHRLICH ascites carcinoma *in vivo* and *in vitro*. The prolongation of the survival time of mice (*in vivo* assay) by macracidmycin was tested: 24 hours after intraperitoneal injection of 2×10^6 cells of EHRLICH ascites carcinoma 0.2 ml of a macracidmycin-containing solution was injected intraperitoneally once daily for 10 days. Cytotoxicity against EHRLICH ascites carcinoma cells in agar plate was assayed by the methods of YAMAZAKI *et al.*[2] and PERLMAN *et al.*[3] (*in vitro* assay).

When *Streptomyces atrofaciens* No. M590-G2 was shake-cultured in a medium containing 1.0% glucose, 1.0% starch, 1.5% partially hydrolyzed soybean meal (Prorich, Ajinomoto Co., Tokyo), 0.1% KH_2PO_4, 0.1% $MgSO_4\cdot 7H_2O$, 0.3% NaCl, Cu^{2+}, Fe^{2+} and Zn^{2+} 2 ppm each, pH 7.2, at 28°C for 72 hours, the 16-fold diluted culture filtrate prolonged over three times the survival time of EHRLICH carcinoma-bearing mice. The culture filtrate was adjusted to pH 7.0 and the active substance was precipitated by addition of ammonium sulfate at 50% saturation. The precipitate was dissolved in 10 volumes of 0.01 M Tris-HCl buffer (pH 7.0) and after removing the insoluble materials by centrifugation, reprecipitated with 50% saturation of ammonium sulfate. A crude brownish powder of macracidmycin was obtained by dialysis of the resulting precipitate against the Tris-HCl buffer followed by lyophilization: a minimum active dose against EHRLICH carcinoma was 62.5 mcg/mouse/day for 10 days.

When this crude powder was subjected to column chromatography using Bio-Gel A1.5 m (50 to 100 mesh, Bio Rad Lab.) equilibrated with 0.05 M Tris-HCl buffer (pH 7.0) containing 0.001 M EDTA and 0.05 M mercaptoethanol, three peaks exhibited cytotoxic activity against EHRLICH ascites carcinoma in agar plate without serum.[2] They were designated FI, FII and FIII. Among these three fractions, only FIII had activity, when tested against the carcinoma cells in agar plate with serum, FI and FII were active against EHRLICH ascites carcinoma cells only in the plate without calf serum but were not active in the plate with serum. The activity in the agar plate with serum was parallel to the activity *in vivo*, and only FIII fraction prolonged the survival time of EHRLICH carcinoma-bearing mice. Since we found that the active substance could be determined by the plate method using carcinoma cell agar with serum, determining this activity we could follow the purification of macracidmycin. The crude powder obtained by ammonium sulfate precipitation was dissolved in 0.02 M phosphate buffer (pH 6.0, PB) and was applied to a DEAE-cellulose column equilibrated with the same buffer. After the column was washed with PB buffer, a stepwise elution was carried out by increasing the concentration of NaCl from 0.05 to 0.4 M in PB buffer. Macracidmycin-containing fractions eluted with 0.05 M NaCl were concentrated by a Dia-Filter G-10 and applied to a CM-Sephadex C-50 column chromatography developed with 0.02 M acetate buffer (pH 6.2). Macracidmycin passed through the column without retention, and the active fractions were combined and lyophilized. The minimum active dose of the resulting powder was 4 mcg/mouse/day for 10 days. This active powder was dissolved in a small amount of deionized water and further purified by gel filtration on a Sephadex G-100 column with deionized water. From the fractions, pure macracidmycin was obtained as white fluffy powder by lyophilization. The macracidmycin in the effluent could be stored in 0.02 M phosphate buffer containing 0.1 M NaCl at 4°C without inactivation.

Macracidmycin is soluble in water but substantially insoluble in organic solvents. It is relatively stable at pH 6 to 10. It exhibits ultraviolet absorption maximum at 277 nm

($E^{1\%}_{1cm}$ 14.3 in water) as shown in Fig. 1. The IR absorption spectrum in Fig. 2 shows its peptide nature. Macracidmycin gives positive FOLIN-LOWRY, ninhydrin, SAKAGUCHI and biuret reactions and negative ELSON-MORGAN, orcinol, phenol-H_2SO_4, ALLEN and anthrone reactions. It has isoelectric point of pH 6.0 by electrofocusing in Ampholine. Macracidmycin has very weakly acidic property as shown by isoelectric point and polyacrylamide gel electrophoresis (7.5 % gel, pH 8.0 and 9.4). Its molecular weight is 35,000~38,000 by Sephadex G-100 and Bio-Gel A 1.5 m gel filtrations. Result of the amino acid analysis of the acid hydrolysate (110°C in 6 N HCl for 20 hours) with the molar ratio of the amino acid given in the parenthesis is as follows:

Fig. 1. Ultraviolet absorption spectrum of macracidmycin in H_2O

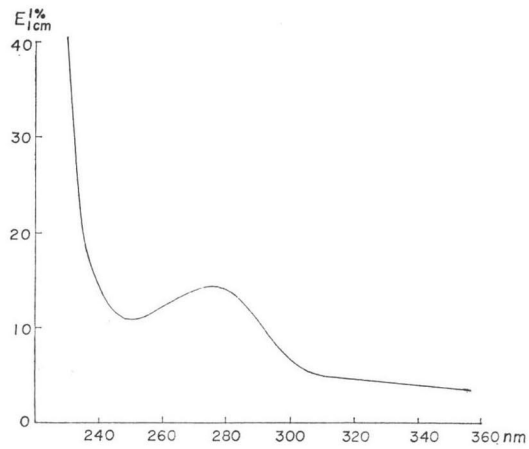
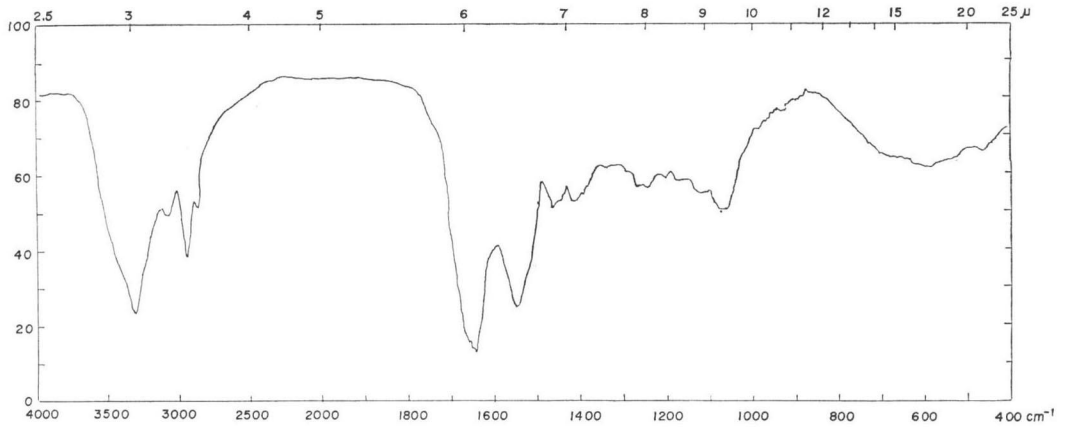


Fig. 2. Infrared absorption spectrum of macracidmycin (KBr)



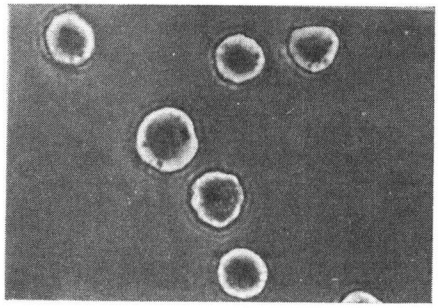
aspartic acid (8), threonine (7), serine (8), proline (2), glycine (16), isoleucine (2), leucine (4), tyrosine (2), phenylalanine (2), glutamic acid (7), alanine (10), valine (4), histidine (1), lysine (3), ammonia (16), arginine (2) and an unknown amino acid between histidine and lysine.

Macracidmycin has no antimicrobial and enzyme activities, and is resistant to proteases. One of the important characteristics of macracidmycin is that it does not inhibit the growth of microorganisms even at a high concentration (100 mcg/ml) but inhibits completely the growth of HeLa S_3, L1210 and L5178Y cells in culture at concentrations of 0.5~2 mcg/ml. Exposure of these cells and ascites cells of EHRLICH carcinoma and sarcoma 180 to a low concentration of macracidmycin such as 0.1 mcg/ml for 1~5 minutes causes swelling of cells (Fig. 3), leakage of amino acids and nucleotides from the pools, and cell lysis. Prolongation of the survival time of EHRLICH ascites carcinoma-bearing mice was observed by daily intraperitoneal injection of 50~1,500 mcg/kg of macracidmycin for 10 days. Thus, the therapeutic index was higher than 30. At doses of 1, 0.5 and 0.25 mg/kg/day for 10 days, all of the tumor-bearing mice fully recovered, and rejected the rechallenge of EHRLICH carcinoma. LD_{50} by a single intraperitoneal injection is 8 mg/kg, and that by intravenous injection is 1 mg/kg in *dd* mice.

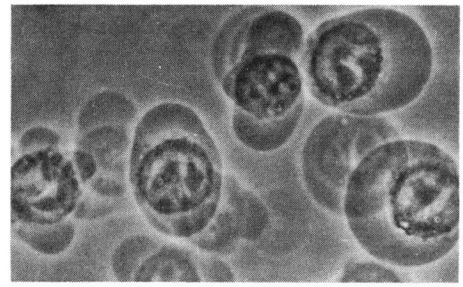
Among known high molecular weight antitumor substances, enomycin,[4] actinocarcin,[5] peptimycin,[6] sanitamycin,[7] phenomycin,[8] lymphomycin,[9] melanomycin,[10] A 216[11] and

Fig. 3, Swelling of EHRLICH ascites carcinoma cells in phosphate buffered saline containing 0.15 M sucrose and 1 mcg/ml macracidmycin

(a) control cells



(b) 10 minutes after addition of macracidmycin



carzinocidin[12] are macromolecular peptides having antitumor activity without antimicrobial action. A 216, enomycin, phenomycin, peptimycin and actinocarcin are different from macracidmycin in their basic properties, molecular weights and amino acid compositions. Lymphomycin and melanomycin which have acidic property are clearly differentiated from macracidmycin in their UV spectrum, isoelectric point and the black color. Sanitamycin is similar to macracidmycin in the acidic property and the molecular weight, but differs in its isoelectric point (pH 6.8) and selective inhibition on the incorporation of ^{14}C-leucine into EHRLICH ascites carcinoma cells. As described above, macracidmycin interferes with membrane function and inhibits the syntheses of protein, RNA and DNA equally. The authors could not find any known substance identical with macracidmycin.

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