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PRODUCTION OF INTERFERON AND OTHER LYMPHOKINES DURING MURINE TUMOUR GROWTH. III. CHARACTERIZATION OF MICROBIAL GROWTH INHIBITORY ACTIVITY IN CELL-FREE SUPERNATANTS OF RAT ZAJDELA ASCITES HEPATOMA

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Summary. — A microbial growth inhibitory factor (MGIF) detected in the cell-free fluid of Zajdela ascites hepatoma of the rat was characterized by means of gel filtration, ion exchange chromatography and isoelectric focusing. It was shown that MGIF is a nondialysable protein with a molecular weight of about 19,000 to 25,000. Charge heterogeneity was revealed by isoelectric focusing which demonstrated pI of 3.8—4.5 and 5.0—6.0. MGIF seems to differ from tumour cell-derived MIF detected in the same ascitic fluids.

Key words: microbial growth inhibitory factor; Zajdela ascites hepatoma

Introduction

Previously (Zschiesche *et al.*, 1980) we described the appearance of soluble factors in cell-free ascitic fluid from rat Zajdela hepatoma which are capable of altering the biological behaviour of macrophages. Similar substances have been found in supernatants of tumour cell cultures (Fauve *et al.*, 1974; Normann and Sorkin, 1977; Rabatic *et al.*, 1977; Nelson and Nelson, 1978), in cell-free tumour ascites (Zschiesche *et al.*, 1976; Lackovič and Borecký, 1979; Lackovič *et al.*, 1980) or could be extracted from tumour cells themselves (Snyderman and Pike, 1976). That such substances might in fact play a role in vivo was shown by Pike and Snyderman (1976).

In the systems studied in our laboratories, interferon-like, migration inhibitory (MIF) and microbial growth inhibitory activities (MGIF) were found in tumour ascitic fluid (Lackovič *et al.*, 1980; Zschiesche *et al.*, 1980). The MIF and MGIF found in ascitic fluid of Zajdela hepatoma-bearing rats seem to be generated by the tumour cells themselves (Zschiesche *et al.*, 1980).

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Table 1. MGIF activities from ascitic hepatoma fluid (AH) found in su pernatants obtained by ammonium sulfate precipitation of cell-free ascitic fluid

Ammonium sulphate saturation	MGIF activity % ± SE
0	61.2 ± 4.8
30%	68.3 ± 8.3
40%	47.1 ± 6.0
50%	22.7 ± 6.4
60%	17.1 ± 8.2
70%	4.4 ± 9.9

Ascites harvested 9 days after tumour inoculation.

Both factors are species nonspecific. The properties of the respective MIF hitherto determined seem to resemble those of the corresponding conventional lymphocyte-derived factor (Fahlbusch *et al.*, 1981). Because MGIF is present in relatively high titres in the ascitic fluid, we decided to study also some properties of the MGIF. In this work we attempted to purify MGIF and to separate it from the MIF-like activity.

Materials and Methods

Animals. Tumour passages were performed in outbred Wistar rats, weighing 70—90 g, obtained from a local breeder. Guinea pigs weighing 300—400 g, from the breeding stock of the Central Institute, were employed as the source of peritoneal macrophages for the migration assay. AB/Jena inbred strain mice, weighing 20—24 g and aged 5—6 weeks, served as source of peritoneal macrophages for the microbial growth inhibitory assay.

Tumour. Zajdela ascites hepatoma (obtained from the Central Institute of Cancer Research, Academy of Sciences of the GDR, Berlin-Buch) was serially passaged intraperitoneally. Ascites was harvested 7—9 days after tumour cell inoculation and centrifuged twice at 800 rev/min. The cell-free supernatants were stored at —20°C until use.

MGIF assay. Microbial growth was determined on mouse peritoneal macrophages and *Corynebacterium murium kutscheri* (S 8507) as described (Zschiesche *et al.*, 1978). Growth inhibition of 30% was considered as significant.

Ammonium sulphate treatment of cell-free ascites supernatants was performed at 4°C. Solid ammonium sulphate was added very slowly to the supernatant fluids until the desired saturation. After centrifugation at 10,000 × g for 10 min at 4 °C, the pellets were discarded and the supernatant collected and sulphate-free dialysed before testing.

Fractionation procedures. Cell-free ascitic fluids collected at days 7 or 8 after tumour challenge were dialysed against phosphate buffered saline (PBS), pH 7.2, and fractionated on columns (1.5 × 90 cm) of Sephadex G-100 (Pharmacia, Uppsala) at 4 °C. Eluations were made with PBS at a flow rate of 9 ml/hr. Absorbancy was recorded at 280 nm on a Uvicord II (LKB, Stockholm) spectrophotometer. Eluates were pooled into appropriate fractions which were tested at dilutions of 1 : 10 or higher (Fig. 1) in Eagle's MEM (Staatliches Institut für Immunpräparate und Nährmedien, Berlin-Weissensee) for bactericidal activity. The following markers (Serva, Heidelberg) were used for column calibration: blue dextran, bovine serum albumin (Kav = 0.476), chymotrypsinogen A (Kav = 0.573), and cytochrome c (Kav = 0.667). For further characterization of MGIF portions of cell-free ascitic fluids were dialysed against 0.01 mol/l phosphate buffer, pH 7.4, and separated by ion exchange chromatography on DEAE-Sephadex A-50 (Pharmacia, Uppsala). After application of the samples, the columns were washed with about 150 ml of 0.01 mol/l phosphate buffer, pH 7.4. Elution was performed with a linear gradient made-up by mixing 500 ml of the starting buffer with 500 ml of 0.01 mol/l phosphate buffer in 0.4 mol/l NaCl at a flow rate of 45 ml/hr. Eluates were pooled into appropriate fractions and extensively dialysed against PBS. For MGIF assay, portions were diluted 1 : 50 and 1 : 100 with medium.

Isoelectric focusing. Twenty µl of crude cell-free ascitic fluid or of fraction II/2 (Fig. 1) were run in 5% flat-bed acrylamide fels containing ampholines in the pH range from 3.5 to 9.5 at 25 W constant power and 10 °C for 2.5—3.0 hr. Then the gel was cut out into 14 fractions, which were extracted either with 0.1 ml of boiled distilled water for pH measurements or with 0.2 ml of PBS for MGIF assay. The latter material was extensively dialysed against PBS (pH 7.2) to remove the ampholines and sucrose, and finally against the medium mentioned above.

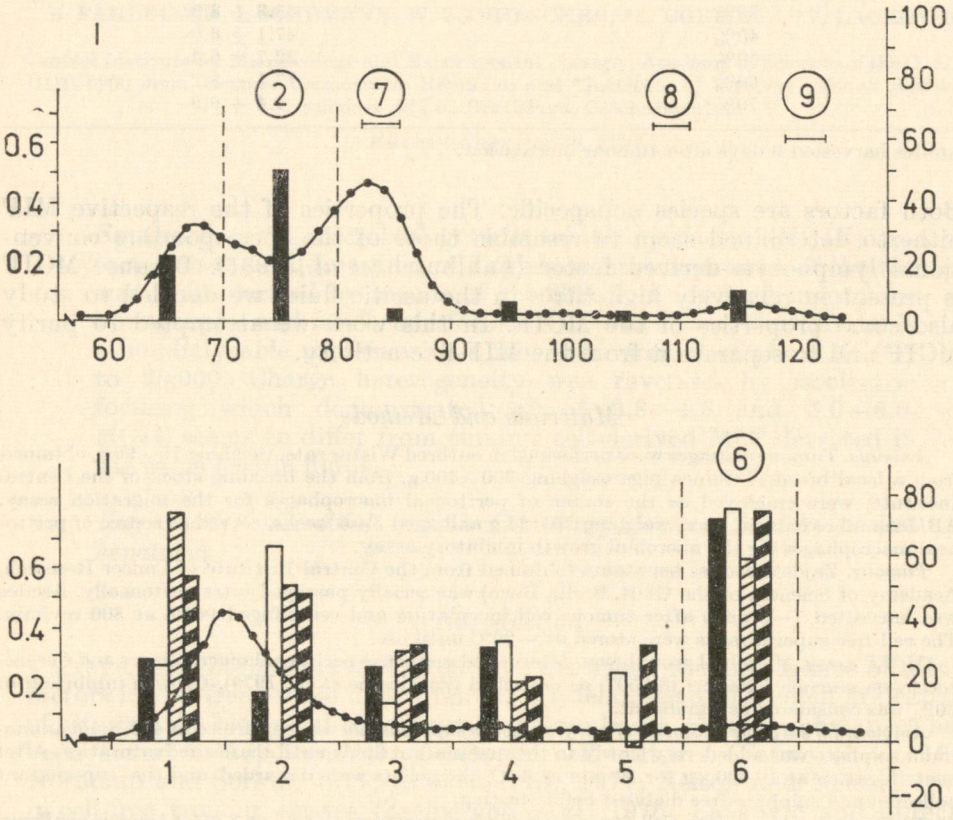

Fig. 1.

Gel filtration of cell-free ascites from Zajdela hepatoma on Sephadex G-100

I — 1 ml ascites fluid was applied to 1.5 × 95 cm column and eluted with PBS. Appropriate fractions (1—6) were pooled and tested at dilutions of 1 : 10 (black columns) for bactericidal activity. Abscissa: elution volume (ml).

II — Fraction I/2 was lyophilized, dissolved in 1 ml of distilled water and rechromatographed on the same column.
Fractions II/1—6 were tested at dilutions of 1 : 50 (black columns), 1 : 100 (empty columns), 1 : 200 (dashed columns, thin lines) and 1 : 400 (dashed columns, thick lines) respectively. Abscissa: fraction numbers; left ordinate: absorbance at 280 nm; right ordinate: % inhibition of microbial growth in macrophages

7 — BSA
8 — chymotrypsinogen
9 — cytochrom c

Results

Ammonium sulphate precipitation

Because of the rather high protein content of the ascitic fluids we attempted to remove the contaminating proteins by ammonium sulphate treatment. MGIF did not entirely precipitate at one certain saturation point but instead was observed in a range up to 50% $(NH_4)_2SO_4$ saturation (Table 1). However,

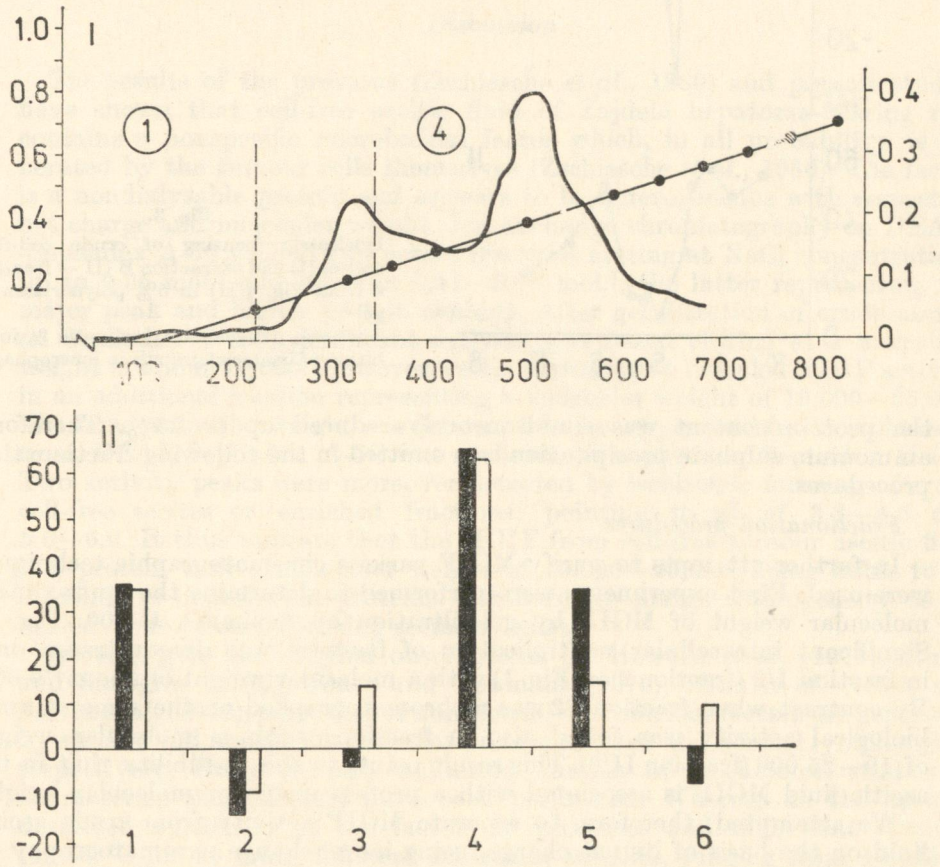

Fig. 2.

Ion-exchange chromatography of the cell-free ascites on DEAE-Sephadex A-50

I — 50 ml ascites fluid was applied to 2.5 × 35 cm column and eluted at increasing NaCl concentrations
Abscissa: elution volume (ml)
Left ordinate: absorbance at 280 nm
Right ordinate: molarity of NaCl

II — Fractions 1—6 tested at dilutions 1 : 50 (black columns) and 1 : 100 (empty columns) respectively for bactericidal activity
Abscissa: fraction numbers; ordinate: % inhibition of microbial growth in macrophages

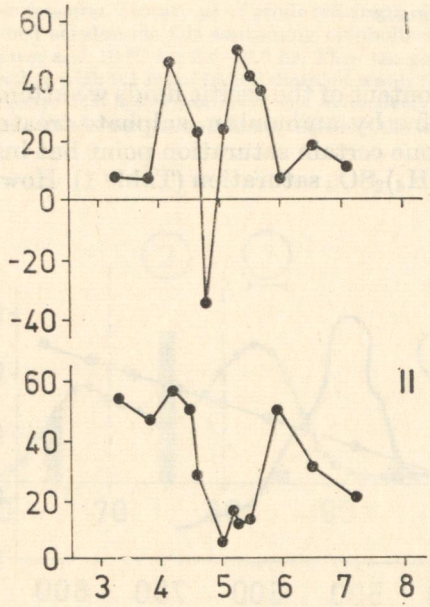

Fig. 3.
Isoelectric focusing of crude cell-free ascites (I) and of fraction B (II — fraction 6 from Fig. 1/II) in 5% polyacrylamide gel
Abscissa: pH-gradient; ordinate: % inhibition of bacterial growth in macrophages

the protein content was simultaneously reduced up to 25%. Therefore, ammonium sulphate precipitation was omitted in the following fractionation procedures.

Fractionation procedures

In further attempts to purify MGIF various chromatographic techniques were used. Firet experiments were performed to determine the approximate molecular weight of MGIF by gel filtration on Sephadex G-100 (Fig. 1). Significant intracellular multiplication of bacteria was demonstrated only in fraction I/2 (fraction 2 on Fig. 1) with a molecular weight of about 70,000. By contrast, when fraction I/2 was rechromatographed on the same column, biological activity was found also in fractions with a molecular weight of 19—25,000 (fraction II/6). This result points to the possibility that in the ascitic fluid MGIF is associated with a protein of higher molecular weight.

We attempted, therefore, to separate MGIF activity from crude ascitic fluid on the basis of its net charge, using ion exchange chromatography on DEAE-Sephadex A-50. Elution was accomplished with a linear gradient of NaCl. A typical elution profile of crude ascitic fluid is shown in Fig. 2. Significant MGIF activity was found in two areas. The first part of the activity (fraction 1) eluted up to 0.05 mol/l NaCl and another one between 0.11—0.1 8 mol/l NaCl (fraction 4). After concentration, fraction 4 (fraction 4 on Fig. 2) was reapplied to a Sephadex G-100 column to separate MGIF activity from contaminating proteins. Based on comparisons with appropriate standards, the active peak was found in a fraction corresponding to a molecular weight of 20—25,000.

Isoelectric focusing

For further characterization and differentiation of the MGIF-like factor from other cytokines, the isoelectric point (pI) of biological activity was determined. Two peaks with significant MGIF activity at pH 3.8—4.5 and 5.0—6.0, being indepedent of the degree of purification of the applied sample, were detected (Fig. 3).

Discussion

The results of the previous (Zschiesche *et al.*, 1980) and present studies have shown that cell-free ascitic fluid of Zajdela hepatoma-bearing rats contains a nonspecific microbicidal factor which, in all probability, is generated by the tumour cells themselves (Zschiesche *et al.*, 1980). The factor is a nondialysable protein and appears to be heterogeneous with respect to net charge and molecular weight. Ion exchange chromatography on DEAE--Sephadex A-50 yielded two active fractions eluting at NaCl concentrations up to 0.05 mol/l and between 0.11—0.18 mol/l, the latter representing the major peak and higher protein content. After gel filtration of crude ascites on Sephadex G-100, significant activity was found eluting at a molecular weight of about 70,000. However, rechromatography revealed MGIF activity in an additional fraction representing a molecular weight of 19,000—25,000. The same molecular weight was determined when the second fraction obtained from DEAE-Sephadex A-50 was rechromatographed on Sephadex G-100. Two activity peaks were moreover detected by isoelectric focusing of crude cell-free ascites or enriched fractions, pointing to pI of 3.8—4.5 and 5.0—6.0. It thus appears that the MGIF from cell-free tumour ascitic fluid is a protein with a molecular weight of 19,000—25,000, being liable to be attached to nonrelevant proteins. Furthermore charge heterogeneity as revealed by isoelectric focusing seems to exist.

According to the original observations by Howard *et al.* (1971), Simon and Sheagren (1972), Jones and Youmans (1972), Klun *et al.* (1973) and Salvin *et al.* (1974), a number of nonspecific microbicidal factors are generated by specific and nonspecific stimulation of sensitized and normal lymphocytes, respectively. However, only a limited number of biochemical studies to characterize those factors have been performed. Whereas all the authors described sensitivity of the factors to proteases and their heat stability (at 56 °C for 30 min), different molecular weights, ranging from 10,000 to more than 50,000, were reported (Fowles *et al.*, 1973; Middlebrook *et al.*, 1974; Cahall and Youmans, 1975; Turcotte *et al.*, 1976; Fahlbusch and Schumann, 1979). It remains to be elucidated whether this divergency is due to different chemical treatment of actually one factor or whether a family of different or related factors is involved.

On the other hand, even the chemical individuality of the MGIF as one factor or a class of factors is still doubtful. Thus a number of activities stimulating certain functions of macrophages and collectively denoted as 'macrophage activating factor' has been described. With regard to the special

test systems used, the following particular factors have been postulated: macrophage migration stimulatory factor (Weisbart *et al.*, 1974); macrophage cytotoxicity factor (Lohmann-Matthes *et al.*, 1973; Fidler *et al.*, 1976); phagocytosis enhancing factor (Donahoe and Huang, 1976); microbicidal factor(s). At least two of them, the macrophage cytotoxicity and phagocytosis enhancing factors, have been claimed to be identical with interferon (Donahoe and Huang, 1976; Schultz and Chirigos, 1978).

As already reported by Salvin *et al.* (1974), no unequivocal separation of MIF and MGIF by physicochemical techniques was achieved so far. However, their different kinetics of generation as referred to above and by others (Simon and Sheagren, 1972; Fahlbusch and Schumann, 1979) point to the existence of two individual factors. The possible dissociation of tumoricidal and microbicidal activities has been suggested by studies on genetically defective mouse strains (Boraschi and Meltzer, 1979).

It has been shown that MIF or MGIF-containing supernatants may also inhibit the intracellular growth of certain viruses in macrophages (Alexandrescu *et al.*, 1974; Schumann *et al.*, 1979). Hence, it remains to be elucidated whether MFIF-like factors bear some relation to immune interferon (IFN-γ). More refined immunological methods, like application of monoclonal antibodies agains lymphokines (Luben and Mohler, 1980), have to be developed to resolve these questions.

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