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PRODUCTION OF INTERFERON AND OTHER LYMPHOKINES DURING MURINE TUMOUR GROWTH. I. LYMPHOKINES IN CELL–FREE FLUID OF RAT ZAJDELA ASCITES HEPATOMA

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Summary. — Cell-free ascites of rat Zajdela hepatoma was assayed for the presence of lymphokine-like factors. Macrophage migration inhibitory and microbial growth inhibitory cytokines were detected with peak activities at days 4–8 and 10–11 after tumour inoculation, respectively. Occasionally, skin reactive activity was found, whereas only borderline titres of an interferon-like substance were demonstrated. Preliminary studies indicated that both migration inhibitory and microbial growth inhibitory factors are proteins resembling the corresponding lymphocyte-derived lymphokines. The cellular site of formation of these factors remains to be determined.

Key words: *rat Zajdela hepatoma*; *cytokines*; *allogeneic tumour cells*

Introduction

In the past decade, a number of soluble factors interfering with macrophage functions have been described to occur in connection with malignant growth. Chemically apparently heterogeneous substances capable of inhibiting macrophage chemotaxis and spreading have been found in tomour cell culture supernatants or cell-free tumour ascites (Wolberg, 1971; Fauve *et al.*, 1974; Brozna and Ward, 1975; Snyderman and Pike, 1976; Fauve and Hevin, 1977; Normann and Sorkin, 1977; Rabatić *et al.*, 1977). They were generally considered to be of tumour origin. On the other hand, there is evidence that also host-derived factors are generated. Some reports have

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pointed to interferon (Lackovič and Borecký, 1970; Svet-Moldavsky *et al.*, 1974; Trinchieri *et al.*, 1978) and lymphokine-like substances acting on macrophages (Zschiesche *et al.*, 1976), ocurring in the course of tumour growth. However, the data on interferon have not been unequivocally confirmed (Gresser, personal communication 1978).

The present studies concerned the demonstration of soluble factors in the cell-free ascitic fluid of allogeneic murine tumours. The first paper deals with the presence and preliminary partial characterization of activities affecting macrophage migration and microbial growth inhibitory and skin reactive activities in rat Zajdela hepatoma ascites.

Materials and Methods

Animals. Tumour passage was performed in outbred Wistar rats, weighing 70—90 g, which were provided by a local breeder. Guinea pigs weighing 300—400 g, obtained 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 5—6 weeks of age, served as source of peritoneal macrophages for the microbial growth inhibitory assay. All the animals were housed in plastic cages and allowed free access to standard food pellets and tap water ad libitum.

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 at intervals after tumour cell inoculation and centrifuged twice at 800 rev/min. The cell-free supernatants, in general as samples pooled from about 5 rats each, were stored at -20 °C until use.

Harvest of macrophages. Guinea pig peritoneal macrophages stimulated by sterile liquid paraffin and mouse peritoneal macrophages stimulated by thioglycollate were harvested by peritoneal lavage as described (Zschiesche *et al.*, 1978).

Table 1. Appearance of macrophage migration inhibitory activities, microbial growth inhibitory activities and interferon in the ascites of Zajdela hepatoma-bearing rats

Days after tumour inoculation	Migration inhibitory activity[1])	Microbial growth inhibitory activity[1])	Interferon units/ml[2])
4	2/2	—	20—40
5	—	0/2	<20
6	3/4	3/5	—
7	4/4	5/10	<20
8	3/4	3/7	<20
9	2/7	10/10	—
10	2/8[3])	6/7	—
11	1/3[3])	4/6	—
12	—	1/1	—
13	—	0/1	—

[1]) Positive samples/total No. of samples. Data compiled from different experiments.
[2]) Data from one experiment.
[3]) One positive sample each exhibited stimulatory activity.

Macrophage migration assay was performed with guinea pig macrophages. The capillary tube technique and calculation of indices were as described (Zschiesche *et al.*, 1978). Test samples were diluted 1 : 10 if not otherwise stated. Migration inhibition of > 20 % was considered as significant.

Skin reactive activity was tested by intracutaneous injection of 0.1 ml of ascites in guinea pigs a described (Zschiesche *et al.*, 1976).

Microbial growth inhibitory assay. Microbial growth was determined using mouse peritonea. macrophages and *Corynebacterium murium kutscheri* (S 8507) as described (Zschiesche *et al.*, 1978)l Ascites samples were tested in a 1 : 10 dilution if not otherwise stated. Growth inhibition of > 30 % was considered as significant.

Interferon assay. Rat interferon was determined as described by Lackovič and Borecký (1970), using primary and passaged rat embryo cells and encephalomyocarditis (EMC) virus for challenge.

Results

Rats routinely inoculated with Zajdela hepatoma showed widely varying survival times from 14–30 days. Preliminary studies demonstrated that, in a range from 5×10^6 to 1×10^8 cells, survival times were approximately inversely proportional to the number of tumour cells inoculated.

Macrophage migration affecting activities were found in many ascitic samples (Table 1). The data indicate the existence of certain temporal kinetics,

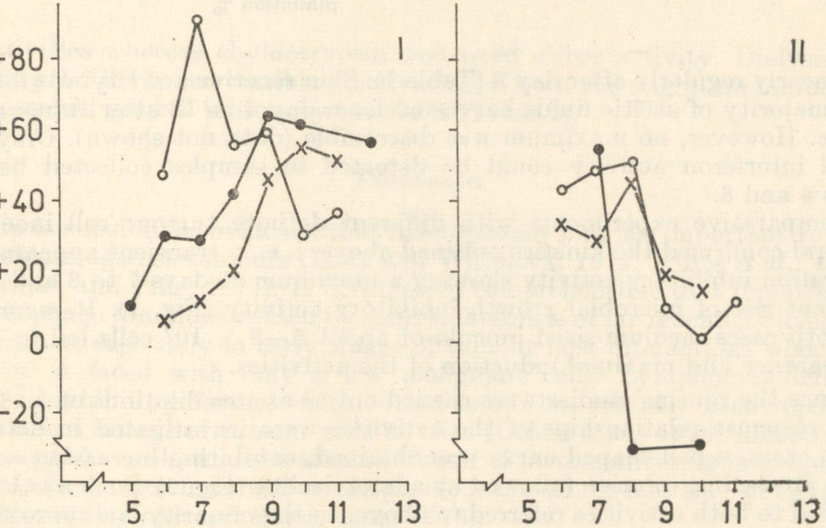

Fig. 1.

Kinetics of microbial growth inhibitory (I) and macrophage migration inhibitory (II) activities in dependence on the size of tumour cell inocula

● $1-4 \times 10^7$ cells
○ $5-8 \times 10^7$ cells
× $>8 \times 10^7$ cells
Abscissa: time in days; ordinate: inhibition %

although they were obtained from mutually independent groups of rats injected with tumour cell inocula of different size. Thus, migration inhibitory activity was detected from days 4 to 8 and subsequently declined. In two cases, migration accelerating activity was found at days 10 and 11. By contrast, microbial growth inhibitory activity was not present until day 6,

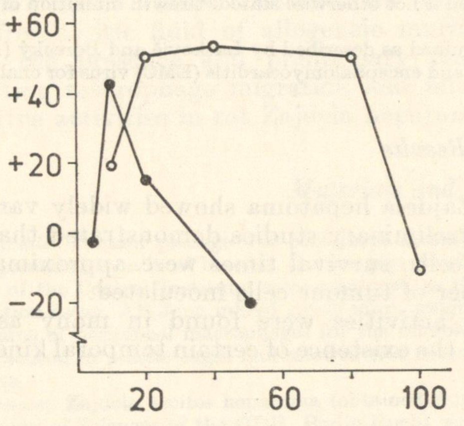

Fig. 2.
Dilution of microbial growth inhibitory (○) and macrophage migration inhibitory (●) activities

Abscissa: dilution reciprocals; ordinate: inhibition %

but nearly regularly after day 9 (Table 1). Skin reactive activity was found in the majority of ascitic fluids harvested from day 4 to 12 after tumour challenge. However, no maximum was discernible (data not shown). Only marginal interferon activity could be detected in samples collected between days 4 and 8.

Comparative experiments with different definite tumour cell inocula in general confirmed the kinetics outlined above, i. e., a transient appearance of migration inhibitory activity showing a maximum at days 7 to 8 and a subsequent rise of microbial growth inhibitory activity (Fig. 1). It seems that in both cases medium-sized inocula of about $5-8 \times 10^7$ cells led to earlier appearance and maximal induction of the activities.

Since the routine studies were carried out at ascites dilutions of 1 : 10, the dose-response relationships of the activities were investigated in detail. In most cases, a bell-shaped curve was obtained, exhibiting increasing activity with proceeding dilution followed by a later decline (Fig. 2). Such a behaviour applied to both activities referred to above. In the minority, an approximately linear dose-dependent decrease with higher dilution was seen.

Migration inhibitory and microbial growth inhibitory activities were found to be heat stable at 56 °C for 30 min. Temperatures up to 100 °C increasingly destroyed either activity, and the latter turned out to be more sensitive than the former. Both activities, lyophilized as crude, cell-free ascites and kept at −20 °C, proved to be stable for at least up to 6 months, in some cases for more than one year. Digestion with bovine ribonuclease failed to affect

Table 2. Characterization of macrophage migration and microbial growth inhibitory factors from ascites of Zajdela hepatoma-bearing rats

Treatment	Migration inhibitory activity before treatment	Migration inhibitory activity after treatment	Microbial growth inhibitory activity before treatment	Microbial growth inhibitory activity after treatment
56 °C, 30 min		24.8 %	48.5 %[1]	38.1 %[1]
80 °C, 30 min	30.0 %	1.0 %		
100 °C, 30 min		4.7 %		
pH 2[2]	30.0 %	14.1 %	36.0 %[1]	13.0 %[1]
Ribonuclease[3]	34.2 %	42.4 %	43.4 %	56.7 %
Chymotrypsin[4]	21.5 %	0.1 %	51.5 %[5]	18.4 %[5]

[1]) Dilution 1 : 5.

[2]) Dialysis for 24 hr against 0.1 M citrate buffer and readjustment to pH 7.2 by dialysis against phosphate buffered saline.

[3]) Ribonuclease A (Serva, Heidelberg) was added to cell-free ascites at a final concentration of 50 μg/ml. The mixture was incubated for 1 hr at 37 °C (pH 7.2) and dialysed against phosphate buffered saline before assay.

[4]) Method according to Kühner *et al.* (1976).

[5]) Dilution 1 : 30.

the activities whereas chymotrypsin destroyed either activity. Dialysis for 24 hr at pH 2 led to irreversible inactivation of both the migration inhibitory and microbial growth inhibitory activities (Table 2).

Discussion

There is substantial evidence that macrophages play an important role in host defense against tumours (Levy and Wheelock, 1974; Hibbs *et al.*, 1977; Snyderman and Pike, 1977). It is therefore comprehensible that impairment of macrophage functions caused by soluble factors is of far-reaching biological consequence, especially in early stages of tumour-host interactions when the organism is faced with only a few malignant cells. Lymphocyte-derived migration inhibitory factor, on the other hand, has recently been reported also to affect kinesis of tumour cells in vitro (Cohen *et al.*, 1978). Respective experiments have shown that administration of factors affecting macrophage functions to syngeneic as well as allogeneic hosts can in fact lead to enhaced tumour growth (Pike and Snyderman, 1976).

The migration and microbial growth inhibitory activities described exhibited certain temporal kinetics. Whereas the former was present up to day 8 after tumour challenge, the latter regularly appeared only after day 8 and could be detected till day 12 when the experiments were terminated. Skin reactive activity failed to show unequivocal kinetics. However, previous gel filtration studies had shown that, in addition to the conventional skin reactive factor, other skin irritating material may be contained in tumour ascites whose action pretends skin reactive factor activity (Zschiesche *et al.*, 1976). No substantial interferon activity could be detected in the ascitic fluids. However, since Lackovič *et al.* (1980), using Ehrlich ascites tumour in mice, found interferon as early as 24 hr after tumour inoculation, such activity might have been overlooked in the present experiments started only 4 days after tumour challenge.

A bell-shaped dilution curve of lymphokine-like factors has been observed (Arvilommi *et al.*, 1978- and corresponds to the so-called pro-zone phenomenon described in case of interferon (Waschke *et al.*, 1979). Although no definite explanation can be given at present, it is assumed that inhibition of the lymphokine-like activities in question at low dilutions is due to simultaneous presence of antagonistic factors (see Table 1).

With regard to the physico-chemical and biochemical data obtained (Table 2), both migration inhibitory and microbial growth inhibitory activities are proteins which act species nonspecifically. Their properties hitherto determined seem to resemble those of the corresponding lymhocyte-derived factors. However, the cellular site of formation of these factors could not be unequivocally determined so far. Experiments on hepatoma cells cultivated in vitro yielded conflicting results. Therefore, the pathogenesis of the formation of the factors referred to above remains obscure.

However, the following possibilities have to be considered. (*1*) The factors are elaborated by lymphoid cells and represent a product of antigenic recognition as proposed by Svet-Moldavsky *et al.* (1974). (*2*) The factors are elicited in the course of a delayed-type hypersensitivity reaction against the tumours. Althouh such a possibility cannot definitely be ruled out, the kinetics of the factors argue against it. However, Postlethwaite *et al.* (1976) could demonstrate the transient appearance of macrophage migration inhibitory and macrophage chemotactic factors in immunized guinea pigs after intraperitoneal antigen administration. (*3*) Production of the above factors by lymphocytes or macrophages is stimulated by constituents of normal or necrotic tumour cells, e. g., double-stranded RNA, proteins, which might act mitogenicaliy on host lymphocytes. (*4*) The above factors are synthesized by tumour cells. It has been postulated that rapidly dividing cells should form cytokines spontaneously as a consequence of high mitotic activity (Tubergen *et al.*, 1972). (*5*) Factors may be induced by oncogenic or passenger viruses contained in the tumour cells used. It has been repeatedly shown that viruses are capable of inducing other lymphokines in addition to interferon (Bigazzi *et al.*, 1975; Poste, 1975; Yoshida *et al.*, 1975).

No explanation can be given for the sequential appearance of the lymphokine-like factors. In this context, it may be of more than formal interest that the same sequence, first interferon, second migration inhibitory factor and later microbial growth inhibitory activity, was observed after challenge of mice with Ehrlich ascites tumour cells (Lackovič *et al.*, 1980) or a single injection of mice with tilorone, a low molecular weight interferon inducer (Zschiesche *et al.*, 1978). Further studies should elucidate whether all the factors under consideration are consecutively synthesized by one cell type or whether the kinetics result from a differentially timed synthesis of factors in different cells.

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