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Inhibition of lymphocyte mitogenesis by factor(s) released from macrophages isolated from ascitic fluid of advanced ovarian cancer patients

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Summary. *Ascitic fluid from women with advanced ovarian carcinomas was shown to contain factor(s) which inhibit(s) T lymphocyte mitogenesis. The factor(s) was (were) demonstrated to be associated with the infiltrating macrophages. The inhibition was reversible and inhibited mitogenesis at some late event in the cell cycle. The inhibitory substance(s) was (were) noncytotoxic, dialyzable, heat-stable at 70° C for 10 min (but unstable at 100° C for 15 min), and partially resistant to protease treatment (55%–70%). Further experiments demonstrated that macrophages isolated from the ascitic fluid of patients with cirrhosis of the liver also released factor(s) which inhibit(s) T lymphocyte mitogenesis. On the basis of our data and data from other investigators, we propose that in advanced human ovarian cancer of epithelial origin, macrophages which infiltrate the ascitic fluid elaborate nonspecific inhibitors of T lymphocyte blastogenesis within the proximal environment, resulting in localized immunosuppression and the subsequent enhancement of metastasis within the peritoneal cavity, the tumor cells themselves being resistant to the cytocidal action of the macrophages due to genetic selection and/or their inherent biochemical ability to circumvent normal immunosurveillance mechanisms. This may account, at least in part, for the rapid metastasis and poor prognosis of human ovarian adenocarcinomas.*

Introduction

Generalized immunosuppression often accompanies oncogenesis as a functional aspect of the disease process [7, 9–11, 13, 19, 24]. In recent years, tumor immunologists have demonstrated that the advanced cancer patient may be immunocompromised, at least in part, due to immunosuppressive factors elaborated by the malignant cells [1, 3, 14, 18, 30, 31]. Many of the immunosuppressive factors released by human malignant cells have been shown to inhibit allogenic lymphocyte mitogenesis [14, 30, 31]. In conjunction with these data, other investigators have demonstrated soluble immunosuppressive factors in the ascitic fluid of patients with peritoneal metastatic malignancies [2, 33]. In addition, Hess et al. [15, 16] have recently demonstrated and partially purified noncytotoxic factors from the cell-free ascitic fluid obtained from women with advanced ovarian cancer, which inhibit T- and B-lymphocyte proliferation.

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The purpose of our investigation was to localize and identify the cellular source of the inhibitors of T-lymphocyte proliferation in ascitic fluid from women with advanced ovarian carcinomas by differential separation of the cellular components of the fluid. Other experiments were designed to study some of the physiochemical and biochemical properties of the inhibitory substances.

Materials and methods

Patients. Women with advanced ovarian cancer located at the State University Hospital, Downstate Medical Center, and Kings County Hospital were used as the subjects for this study. None of the patients had undergone chemotherapy or radiotherapy for at least 1 month prior to their selection. The identification and grading of all solid malignant tissue was performed by the Department of Surgical Pathology. The cellular components of the ascitic fluid were identified by the Department of Surgical Pathology and by our laboratory in the Department of Pharmacology.

Ascitic fluid. Fluid was collected (1 l/5 ml heparin) in sterilized containers by paracentesis or at laporatomy and immediately sent to the laboratory for processing. Whole cells were isolated by centrifugation at 800 *g* for 15 min at 4° C in a Sorvall centrifuge. The cell-free ascitic fluid was frozen and either stored or lyphilyzed and then stored. The whole cells were washed with saline–0.01 *M* phosphate buffer: pH 7.4. Erythrocytes were lysed by washing with 0.14 *M* ammonium chloride. Samples with copious amounts of blood or mucin droplets (observed under the light microscope) were not employed in these studies. The cells were washed with the saline–phosphate buffer until a white precipitate was obtained. Then 1 ml cells in buffer was placed on top of 9 ml of a continuous Isolymph gradient (3%–6% Ficoll–Triosil; Pharmacia, Uppsala, Sweden). The gradient was centrifuged at 1,000 *g* for 20 min at 25° C. The fractions containing the individual cellular components were removed with a Pasteur pipet, centrifuged, and washed with the saline–phosphate buffer. Histological identification of the cellular components was performed by light microscopy. Each individual cellular component was then homogenized in medium RPMI 1640 (Gibco, Grand Island, NY) using a Ten Broeck homogenizer. Protein was determined by the method of Lowry et al. [20]. After Ficoll–Triosil gradient centrifugation, the macrophage-enriched fraction was either subjected to 10%–20% gradient centrifugation using an SW27 swinging-bucket rotor

at 20,000 *g* for 2 h in a Beckman ultracentrifuge, or incubated for 45 min at 37° C in serum-free Eagle's basal medium (1–20 × 10^6 cells/10 ml) on conditioned petri dishes (Falcon, Oxnard, Calif). These procedures were instituted for further purification of the macrophages. In the latter method [28], the nonadherent cells were removed by washing with medium, and the macrophages recovered after being incubated with 1 m*M* EDTA in phosphate-buffered saline (pH 7.4) for 30 min. Macrophages were identified by their typical morphology, their ability to imbibe neutral red (0.03% in Eagle's medium), and their phagocytic activity in the presence of 1 : 20 diluted latex particles for 60 min (Difco, Detroit, Mich) or in the presence of antibody-coated sheep erythrocytes [22].

Lymphocytes. Peripheral blood lymphocytes from normal donors were isolated and purified by centrifugation over a Ficoll–Triosil gradient [4]. Cultures consisted of 2 × 10^6 cells per 2 ml medium RPMI 1640 supplemented with 10% fetal calf serum, 1% glutamine, 200 U penicillin, 200 µg streptomycin, and 0.5 µg fungizone (Sigma, St. Louis, Mo). The cultures were then incubated with concanavalin A (Con A; Pharmacia, Uppsala, Sweden) to stimulate lymhocyte mitogenesis. The concentration of Con A utilized was determined from a series of standard curves characterizing each preparation of Con A.

Inhibition of T-lymphocyte mitogenesis. The Con A-stimulated lymphocytes were cultivated with and without varying concentrations of the cellular extracts at 37° C in a 95% O_2–5% CO_2 atmosphere for 64 h in loose-fitting sterilized polyethylene tubes. At this time, 1 µCi ^{3}H-thymidine (280 mCi/mmol; New England Nuclear, Boston, Mass) was added, and the incubation continued for 5 h longer. The cells were then harvested on premoistened glass fiber filters (grade 934 AH; Reeve Angel, Clifton, NJ) washed twice with saline and once with alcohol : ether (3 : 1). After drying, the filter papers were placed in scintillation fluid and counted in a Beckman LS230 liquid scintillation counter. Appropriate corrections for quenching and efficiency were used for all calculations. Each individual assay was performed in triplicate.

Results

Normal T-lymphocyte mitrogenesis

Figure 1 is an illustration of T-lymphocyte proliferation with and without stimulation by the lectin Con A. After an initial lag of 24 h there was a dramatic increase in the growth rate until 96 h. After this time period there was a leveling off of growth. All other experiments in our study, unless specifically noted, used Con A-stimulated lymphocytes incubated in growth medium for 64 h.

Inhibitor activity of the cellular components isolated from ascitic fluid

Initial experiments in our laboratory demonstrated a direct relationship between the amount of inhibitor activity found in the cell-free ascitic fluid and the amount of time the fluid remained in the laboratory before being used. As a typical example, if the whole cells in the ascitic fluid were centrifuged down and separated from the fluid within 15 min of collection of the sample from the patient, the cell-free ascitic fluid contained only 10%–20% of the inhibitor activity of the cell-free ascitic fluid which had been allowed to remain 24 h in the laboratory before the whole cells were separated out. Based on these results, we concluded that the inhibitory activity in the cell-free ascitic fluid originates from cellular components. These data held true for 11 of the 14 ascitic fluid samples we examined. Two of the samples had 80%–90% of

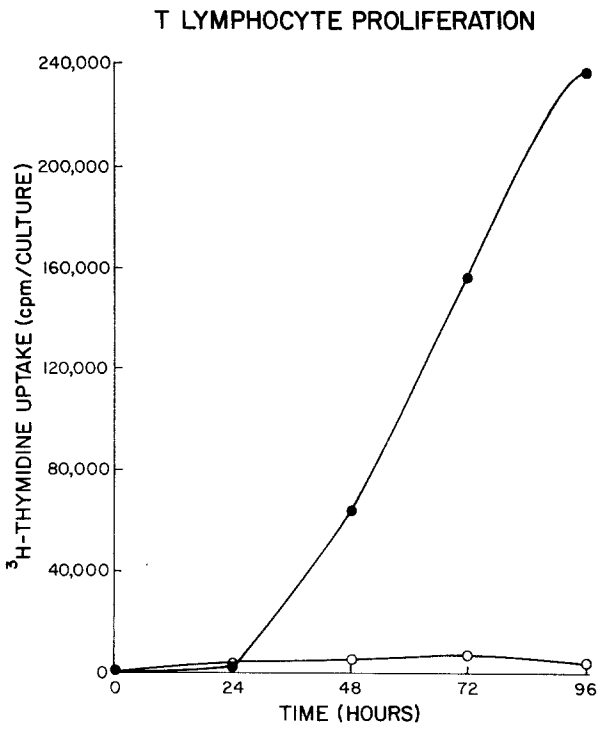

Fig. 1. T-lymphocyte proliferation. T lymphocytes were incubated for 64 h in growth medium. Blastogenesis was determined by measuring ^{3}H-thymidine incorporation

Table 1. Inhibition of T-lymphocyte mitogenesis by cellular components isolated from ascitic fluid obtaibed from women with advanced ovarian cancer of epithelial origin

Fraction[a]	Cell types	Approximate proportion[b]	% inhibitory activity[c, d]
1	Cellular debris	–	2 ± 1
2	Lymphocytes	100	4 ± 2
	Neutrophils	1	
	Plasma cells	1	
3	Lymphocytes	5	57 ± 10
	Mesothelial cells	60	
	Macrophages	40	
4	Mesothelial cells	80	26 ± 7
	Macrophages	20	
5	Mesothelial cells	10	11 ± 4
	Adenocarcinoma cells	0.5	

[a] 90 µg protein from Fractions 2, 3, and 4 were used, while 40 µg protein from Fraction 1 and 5 were used in all assays

[b] The actual number of cells in Fractions 2, 3, 4, and 5 varied widely, depending on the ascites fluid used. As an example, Fractions 2, 3, and 4 contained 1–10 × 10^7 cells, while Fraction 5 had 1–10 × 10^5 cells

[c] The mean standard errors were obtained from the assays of five individual ascitic fluids

[d] Recoveries of inhibitory activity from unfractionated cells were 83%–91%

the total inhibitory activity in the cell-free fluid, even though the samples were processed within 15 min, while one sample had no inhibitor activity in either the cell-free fluid or in the isolated cellular components.

To allow the origin of the inhibitor activity in the ascitic fluid to be discerned, whole cells were isolated and separated into individual cellular components. Table 1 illustrates the separation of the various cellular components of ascitic fluid in a Ficoll gradient. The numerical amount of cells found in each of Fractions 2, 3, and 4 was approximately the same (± 20%). Fraction 5 contained about 10% of the total number of cells found in the other fractions. The cellular debris and Fraction 2, which contained almost exclusively small, well-definied lymphocytes, had only trace amounts of inhibitor activity. Fraction 3, which contained approximately 60% mesothelial cells and 40% macrophages, had 57% of the inhibitor activity. Fraction 4, which consisted of 50% mesothelial cells and 20% macrophages, had 26% of the inhibitor activity. Since Fraction 4 had more mesothelial cells than Fraction 3 and, correspondingly, less inhibitor activity, it is apparent that the inhibitor activity is associated mainly with the macrophages. In support of this conclusion, it can be seen from Table 1 that Fraction 3 had twice the macrophage content of Fraction 4 (each fraction has approximately the same number of whole cells) and correspondingly twice the percentage of inhibitor activity. These same data were consistent for all five of the individual ascitic fluid samples we subjected to Ficoll gradient centrifugation. It should be noted that it is difficult to compare the inhibitory activity in Fractions 3 and 4 with the unfractionated ascitic fluid. The unfractionated ascitic fluid contains numerous other components, including large amounts of albumin which absorb to the substrate and possibly the inhibitory factor(s), which interfere with the biochemical assays. Comparative data would be more than likely to be misleading. Fraction 3 was also subjected to10%–20% sucrose gradient centrifugation using an SW27 swinging-bucket rotor at 20,000 *g* for 2 h in a Beckman ultracentrifuge. A well-defined fraction containing 70% macrophages and 30% mesothelial cells was one of four fractions obtained that contained varying percentages of macrophages and mesothelial cells. This fraction had almost twice the inhibitor activity per microgram of protein than the original Fraction 3, which contained 40% macrophages and 60% mesothelial cells. In other experiments, the macrophage-enriched fraction was incubated in Eagle's basal medium on conditioned petri dishes as described in the *Methods* section. The adherent cells which were recovered were 80%–85% well-defined macrophages and contained approximately 2.5 times more inhibitor activity per microgram of protein than the original Fraction 3, which contained 40% macrophages. This provides further evidence that the inhibitor of T-lymphocyte mitogenesis is associated with the macrophages. Fraction 5, consisting of mesothelial cells and a small number of carcinoma cells, which were usually clumped and difficult to estimate, had 11% of the inhibitory activity. If one assumes that the inhibitor activity was not due to the mesothelial cells, then it appears that the carcinoma cells can elaborate their own inhibitor factor(s). However, there were too few carcinoma cells present to determine whether this is correct. The inhibitory activity of the carcinoma cells may actually originate from leaky or damaged macrophages and be absorbed by the carcinoma cells. It would be of interest to isolate larger amounts of pooled carcinoma cells and determine whether indeed they elaborate their own inhibitor(s) of T-lymphocyte proliferation, and whether this (these) factor(s) is (are) biochemically distinct from the factor(s) released by the macrophages. Initial experiments with solid ovarian tumors used in an attempt to solve this problem have thus far proved to be inconclusive.

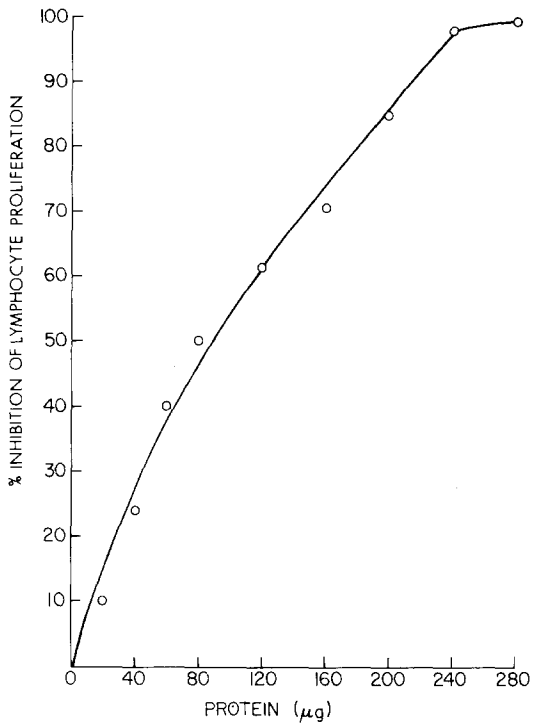

Fig. 2. Inhibition of T-lymphocyte mitogenesis by varying amounts of macrophage-associated inhibitory factors isolated from ascitic fluid

Figure 2 demonstrates that the inhibitor activity derived from Fraction 3 (see Table 1) was dose-respondent, the inhibition being proportional from 20–100 μg protein. For this particular experiment, Fraction 3 from two separate ascitic fluid samples was pooled.

Properties of the inhibitor

The inhibitory factor(s) derived from three different ascitic fluid samples was shown to be noncytotoxic, as demonstrated by the viability of the T lymphocytes using trypan blue exclusion as our test method. The inhibition of T-lymphocyte mitogenesis was also shown to be reversible. This was accomplished by incubating the inhibitory factor(s) derived from three different ascitic fluid samples with lymphocytes for 64 h, at which time the lymphocytes were then reisolated, washed with buffer, and then reincubated with fresh medium and Con A. Under these conditions the lymphocytes proliferated freely with similar growth kinetics to those previously recorded for lymphocytes without the presence of inhibitor (see Fig. 1).

Table 2 shows that the inhibitor can be effectively added 48 h after the stimulation of the lymphocytes with Con A and still elicit a 100% inhibition of lymphocyte mitogenesis. In addition, increased amounts of ^{3}H-thymidine or Con A did not abrogate the inhibition. Therefore, it is apparent that the inhibitor functions at some late event in lymphocyte mitogenesis, and thus does not inhibit Con A binding, thymidine incorporation, or the influx of calcium or carbohydrates into the lymphocytes, which are characteristics of the G_1, phase of lymphocyte proliferation. Other experiments showed that the inhibitor was dialyzable, heat-stable at 70° C for 10 min, but

Table 2. Inhibition of T-lymphocyte mitogenesis after addition of the macrophage-associated inhibitor(s) at varying times after Con A stimulation T-lymphocytes[a]

Time of addition of inhibitor(s) to culture Medium[b]	Percent inhibition
0 h	100
24 h	100
48 h	100
60 h	35
64 h	5

[a] Lymphocytes were routinely incubated for 64 h after stimulation with Con A

[b] Fraction 3 (see Table 1) was used as the source of macrophage-associated inhibitors

unstable at 100° C for 15 min. It was partially resistant (55%–70%) to prolonged trypsin or proteinase K (Sigma, St. Louis, Mo) treatment. Gel electrophoresis, with 10% acrylamide gels (Eastman Kodak, Rochester, NY) showed that the dialysate collected from the inhibitor, then frozen and concentrated by lyophilization, had at least six diffuse bands that were ninhydrin-positive. Therefore, these data do not eliminate the possibility that the inhibitor is a small-molecular-weight polypeptide. At the present time we have not been able to determine whether the inhibitory factor(s) consist(s) of one or many different factors. Studies to determine the complexity and chemical composition of the inhibitory substance(s) are in progress.

Ascitic fluid from other sources

A fraction containing macrophages from ascitic fluid removed from two patients with cirrhosis of the liver also had inhibitor activity (approximately 70%–85% per microgram of protein of the inhibitor activity of macrophages from ascitic fluid obtained from ovarian adenocarcinoma patients). These data, and previous studies which showed that ascitic fluid from patients with benign ovarian neoplasms have inhibitor activity [15, 16], demonstrate that the inhibitor is not specific for tumor-associated macrophages.

Discussion

We have presented data to show that macrophages, which are a major component of the lymphoreticular infiltrate of tumors in humans [5, 8], are the main source of the inhibtory factor(s) of T-lymphocyte mitogenesis found in the ascitic fluid of women with advanced ovarian cancer [15, 16]. The inhibitor(s), which is (are) also found in non-tumor-associated macrophages, did not interfere with an initial event in lymphocyte proliferation such as lectin stimulation, thymidine uptake, or the influx of calcium or carbohydrates into lymphocytes, but rather with some undetermined late event in its cell cycle. The inhibitory substance(s) from tumor-associated macrophages was (were) noncytotoxic, dialyzable, heat-stable at 70° C (but unstable at 100° C), and partially resistant to protease treatment. To determine and the chemical composition and physiochemical properties of the inhibitor(s) and characterize them correctly, it will first be necessary to partially purify the substance(s). It should be noted that to achieve this goal it will be more convenient to isolate the inhibitor from whole cells rather than from cell-free ascitic fluid. The cell-free fluid contains copious amounts of albumin, which can bind strongly to the inhibitor(s), and thus have negative effects on the purification and subsequent charaterization of the biochemical and physiochemical properties of the inhibitor(s). The biochemical properties of the inhibitory factor(s) from the ascitic fluid from patients with cirrhosis of the liver have not been determined. Before these studies are initiated, it will first be prudent to purify the inhibitory factor(s) from tumor-associated macrophages and non-tumor-associated macrophages before all their biochemical and physiochemical properties are delineated, so that meaningful comparative data can be obtained. Comparison of impure preparations of inhibitory factor(s) from different sources may give misleading results.

There has been much evidence indicating that the macrophage content of tumors is related to tumor immunogenicity and metastasizing potential [6, 25] and most tumor-associated macrophages have the potential to express cytostatic activity on tumor cells [6, 25]. However, some investigators have shown that macrophages isolated from ascitic fluid from ovarian cancer patients have lower cytocidal activity against some tumor lines than peritoneal macrophages from patients with benign gynecological disorders [22]. In addition, some ovarian tumors proved to be resistant to macrophage cytocidal action [22, 28]. Moreover, other investigators have demonstrated that peritoneal macrophages from experimental animals can enhance the growth of lymphomas growing poorly in culture [17, 26, 27]. Also, macrophages from two different sarcomas have been shown to enhance tumor cell proliferation in vitro [21, 23, 29], and a relationship between macrophage content and increased metastasis in lung carcinomas has been demonstrated [12]. These latter data have led to the proposal that tumor-associated macrophages may, in some malignancies, stimulate tumor metastasis and/or proliferation in vivo [22, 32]. Our results are in accord with these data.

Additional data demonstrating that the inhibitory factor(s) from macrophages isolated from ascitic fluid obtained from patients with benign ovarian disease [16] and cirrhosis of the liver may indicate that macrophages, in general, contain this inhibitory factor(s). In agreement with this postulate are preliminary (unpublished) experiments from our laboratory that show that peripheral blood monocytes from normal and tumor patients also have inhibitor(s) of T-lymphocyte mitogenesis. However, at this juncture it may be theorized that only after the stimulated release of the macrophage-associated inhibitor(s) of T-lymphocyte blastogenesis does this factor(s) play a significant role in depressing immunogenicity.

We propose that, in advanced human ovarian cancer, infiltrating macrophages in the ascitic fluid elaborate nonspecific inhibitor(s) of T-lymphocyte blastogenesis within the proximal environment, resulting in localized immunosuppression and the subsequent rapid metastasis within the peritoneal cavity (which has now evolved into a privileged site for tumor growth). The ovarian tumor cells themselves are resistant to the cytocidal action of the macrophages due to genetic selection and/or their inherent biochemical ability to circumvent normal physiological immunosurveillance mechanisms [22, 23, 28]. Therefore, the poor prognosis for human ovarian adenocarcinomas may ironically be due, at least in part, to tumor tolerance of the host's immune system, and the subsequent subversion of the host's immune system to enhance the metastasis of the tumor.

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