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Host-Tumor Interaction in Ovarian Cancer

Spontaneous Release of Tumor Necrosis Factor and Interleukin-1 Inhibitors by Purified Cell Populations from Human Ovarian Carcinoma *in Vitro*¹

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The biological activity of tumor necrosis factor (TNF α/β) and interleukin-1 β (IL-1 β) can be blocked by soluble, naturally occurring molecules—TNF α/β binding proteins (BP-55 and BP-75), derived from the extracellular portion of the 55- and 75-kDa TNF α/β membrane receptors, and IL-1 receptor antagonist (IL-1ra), respectively. We examined the levels of these cytokines and their inhibitors in cell-free ascites of 18 patients with advanced ovarian carcinoma by ELISA. Levels of both TNF BP and IL-1ra dramatically exceeded those of TNF and IL-1; thus, it is unlikely that these cytokines are active in ascites from patients with this disease. We then elutriated solid tumor samples from three additional patients, yielding pure populations of tumor cells, macrophages, and lymphocytes. Cells were cultured for up to 48 hr and the spontaneous production of TNF, IL-1, and their inhibitors was measured by ELISA. Tumor cells and macrophages both released inhibitors for TNF and IL-1. Tumor cells released IL-1ra and BP-55, while macrophages released IL-1ra and BP-75. Kinetic studies showed that both tumor cells and macrophages produced an initial burst of TNF α and IL-1 β which was overtaken within 48 hr by a sustained production of TNF BP and IL-1ra. Lymphocytes released no TNF α or TNF β , which alone suggests that tumor associated lymphocytes are locally quiescent *in vivo*. TNF and IL-1 inhibitors originate from tumor cells and tumor associated macrophages and probably block TNF and IL-1 activity locally and regionally in ovarian carcinoma patients. Whether this phenomenon contributes to

the pathogenesis of this disease remains to be determined.

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INTRODUCTION

Ovarian carcinoma is thought to arise from genetic derangements that lead to dysregulation of cell growth and development of invasive and metastatic potential [1-3]. Cytologically, ovarian carcinomatous ascites and solid tumors are infiltrated by both lymphocytes and macrophages [4]. It has been suggested that the uncontrolled growth of this tumor may involve failure of cell-mediated antitumor activity, perhaps through blockade of principal cytokines [5].

Tumor necrosis factor α (TNF α)³ and lymphotoxin (LT/TNF β) are structurally and biologically related cytokines [6] produced principally by activated macrophages [7] and lymphocytes [8], respectively. There is abundant evidence that these cytokines are important in orchestrating host antitumor reactions *in vivo*, not only through direct cytotoxicity, but also through augmentation of tumor antigenicity, recruitment of host lymphoid cells, activation of infiltrating effector cells by autocrine or paracrine pathways, and induction of a cascade release of other cytokines [9]. Interleukin-1 (IL- α , IL- β) is a cytokine which shares many biologic properties with TNF, including antitumor activity [10]. In addition, IL-1 and TNF have demonstrated synergy in lymphoid cell activation and inflammatory reactions, both *in vitro* and *in vivo*.

TNF and IL-1 exert their effects through binding to specific cell surface glycoprotein membrane receptors [6,10]. A new concept has emerged that cells may regulate

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³ Abbreviations used: IL-1ra, interleukin-1 receptor antagonist; TNF BP or BP, tumor necrosis factor binding protein; CFA, cell-free ascites; OC, ovarian carcinoma; TNF α , tumor necrosis factor α ; LT, lymphotoxin.

the biologic activity of the secretory forms of these cytokines through the release of specific inhibitors which block the interaction of cytokines with their cell surface receptors. These inhibitors, in general, are either the shed extracellular portions of specific membrane receptors or secreted, biologically inactive, cytokine homologues which compete for membrane receptor binding sites. Soluble protein inhibitors of TNF bioactivity were initially identified in human urine and serum in both physiologic and pathologic conditions [11–15]. Recently, investigators in our laboratory identified such TNF blocking activity in the sera and cell-free ascites (CFA) from women with ovarian carcinoma and other malignancies [16,17]. This blocking activity was associated with soluble 30- and 40-kDa fragments from the extracellular domains of the 55- and 75-kDa TNF membrane receptors, otherwise referred to as blocking proteins (TNF BP, BP-55, BP-75) [18]. BP-55 and BP-75 were identified by ELISA in CFA as well as in supernatants from short-term cultures of ovarian carcinomatous ascites cells and cell suspensions generated from ovarian carcinoma tissue [18]. It has been shown that TNF BP may sterically bind to TNF, and such mechanical blockade inhibits TNF biologic activity both *in vitro* and *in vivo* [19]. The IL-1 receptor antagonist (IL-1ra), a naturally occurring molecule structurally related to IL-1, inhibits the binding of IL-1 to its cell surface receptors and has been found to block IL-1 activity *in vitro* and *in vivo*; its association with human malignancy is currently being explored [10]. It is logical to assume that the stoichiometric ratio of cytokine to cytokine inhibitor could determine the state of immunologic reactivity both systemically and locally in patients with malignancy.

This study was designed to investigate further the role and interaction of the cytokines TNF and IL-1 and their inhibitors in the ovarian carcinoma disease process. Specifically, we (a) examined intraperitoneal immunologic activity by measuring levels of TNF α/β , IL-1 β , and their inhibitors in CFA from patients with ovarian carcinoma, (b) identified the cells from solid tumor tissue responsible for their spontaneous release *in vitro*, and (c) determined relationships among TNF, IL-1, and their inhibitors in supernatants from short-term cultures of fresh solid ovarian carcinoma-derived lymphocytes, macrophages, and tumor cells.

MATERIALS AND METHODS

Patients and Samples

Samples of malignant ascites were obtained from 18 patients with advanced ovarian carcinoma at the time of diagnostic/staging laparotomy. Fresh samples were rendered cell-free by centrifugation at 3000 rpm for 10 min

in a CR6000 centrifuge (International Equipment Co.), aliquoted, and stored at -20°C . Samples of solid malignant ovarian carcinoma (OC1–OC3), ranging in size from 10 to 20 g, were obtained from the peritoneal cavities of three additional patients, all with Stage IIIC adenocarcinoma, at the time of diagnostic/staging laparotomy. These tumor samples were placed immediately in sterile complete media consisting of RPMI 1640, 15% FCS, and 1% Pen/Strep, 1% L-glutamine (Gibco, Grand Island, NY) in LPS-free sterile water.

Tumor Processing

Solid tumor samples, received in complete media within 4 hr of staging laparotomy, were processed under sterile conditions at room temperature. Grossly viable tumor was mechanically minced in RPMI 1640 to portions no larger than 1 mm³ and washed twice with RPMI 1640. For enzymatic dissociation, portions of minced tumor, not exceeding 5 g, were placed in 250-ml trypsinizing flasks with 30 ml of enzyme solution containing 0.14% collagenase Type I (Sigma) and 0.01% DNase (Sigma, 2000 K units/mg) in RPMI 1640, and incubated on a magnetic stirring apparatus overnight at 4°C. Enzymatically dissociated tumor was then filtered through 150- μm nylon mesh to generate a single cell suspension. The resultant cell suspension was then washed twice in complete media.

Centrifugal Elutriation

Cell separation was performed utilizing a JE-6B elutriation system and rotor with a Sanderson-type chamber in a J2-21 centrifuge (Beckman Instruments, Inc.). After assembly and installation, the rotor was sterilely prepared by infusing the following solutions in sequence using an RP-G50 peristaltic pump (FMI) at maximal speed: 500 ml 10% bleach, 500 ml sterile water (McGaw sterile water for irrigation USP), 500 ml 70% ethanol, 500 ml sterile water, 500 ml sterile phosphate-buffered saline (PBS), and 200 ml elutriation buffer, consisting of RPMI 1640, 2% FCS, prepared with LPS-free sterile water. With rotor speed calibrated to 4000 rpm, a suspension containing 10⁸ cells in 20 ml elutriation buffer was infused at a constant flow rate of 30 ml/min. For the first two tumors processed (OC1 and OC2) the rotor speed was decreased in a step-wise fashion in 100 rpm increments to a final speed of 1600 rpm, and at each speed, 100 ml of eluate was collected. Based on the characteristics of fractions obtained at each centrifuge speed in the first two trials, the protocol was simplified for the third tumor processed (OC3), in which 500 ml fractions were obtained at 4000, 3600, 3200, 2800, 2000, and 1600 rpm. Any cells retained in the Sanderson chamber were transferred to a sterile 50-ml polypropylene tube (Dow Corning) containing elu-

triation buffer. Cell suspensions were centrifuged at 1500 rpm for 10 min, after which pellets were resuspended in 2 ml of complete medium.

Cell Counts and Viability Determination

Cell counts and viability were determined for the original cell suspension as well as for each elutriated fraction, prior to cell culture. Viability was also determined at 24 hr of culture for OC1 and OC2 and at 6, 12, 24, and 48 hr for OC3. Ten microliters of each cell suspension were combined with 90 μ l 0.2% trypan blue. Cell count and viability were determined prior to culture and after specified time points by light microscopy using a hemacytometer, with viability defined as the ability for individual cells to exclude trypan blue. For each tumor, change in viability was defined as the difference between the preelutriation viability and the average viability of elutriated fractions, adjusted for cell density. There was no loss of viability from the elutriation procedure. The initial mean (\pm standard deviation) percentage viability for elutriated fractions was $86 \pm 10\%$ (OC1), $79 \pm 9\%$ (OC2), and $97 \pm 2\%$ (OC3). The mean (\pm standard deviation) percentage decrease in viability after 24 hr culture for elutriated fractions was $10 \pm 8\%$ (OC1), $41 \pm 18\%$ (OC2), and $1.3 \pm 2.3\%$ (OC3). The mean (\pm standard deviation) percentage decrease in viability after 48 hr culture for elutriated fractions was $22 \pm 13\%$ (OC3).

Cell Morphology Characterization

Morphology was characterized for the original cell suspension as well as for each elutriated fraction. Cells (1×10^5) were loaded in a Cytofunnel chamber (Shandon) and spun onto a glass microscope slide in a Cytospin 3 centrifuge (Shandon) at 800 rpm for 5 min. The cytospin preparation was then stained with Wright-Giemsa or hematoxylin-eosin, and morphology was assessed by light microscopy, using a differential grid to determine the percentage of different cell types. For all three tumors, morphologic characterization was performed independently and in a blinded fashion by a board-certified pathologist.

Cell Culture

After centrifugal elutriation and morphologic characterization, cells from both original suspensions and elutriated fractions were cultured at 37°C, 5% CO₂, in 1 to 2 ml complete medium at a density of 1×10^6 cells/ml in 3-ml polypropylene culture tubes. For kinetic studies, individual cultures were incubated for predefined periods, after which homogeneous cell suspensions were examined

TABLE 1
Levels of Cytokines and Cytokine Inhibitors in Malignant Human Ovarian Carcinomatous Ascites

	Cytokine/ inhibitor	Mean concentration (ng/ml)	Standard deviation (ng/ml)	Range (ng/ml)
Cytokine	TNF α	0	0	—
	LT/TNF β	0	0	—
	IL-1 β	0.003	0.006	0-0.020
Inhibitor	BP-55	2.6	0.5	1.9-3.4
	BP-75	4.3	0.5	3.6-5.3
	IL-1ra	1.2	1.6	0.04-7.4

Note. CFA samples derived from 18 patients with histologically proven advanced ovarian carcinoma at the time of original diagnosis were analyzed for the presence of TNF α , LT/TNF β , IL-1 β , BP-55, BP-75, and IL-1ra by ELISA, as described under Materials and Methods.

for viability and centrifuged at 1500 rpm for 10 min. Cell-free supernatants were aspirated and stored at -20°C .

ELISA

Supernatants from 24-hr cultures were collected for OC1-OC3 and from 6-, 12-, 24-, and 48-hr cultures for OC3 only. Concentrations of cytokines and cytokine inhibitors were determined by ELISA, employing commercially available kits for TNF α , TNF β , IL-1 β , and IL-1ra (Research & Diagnostic Systems) or our own polyclonal rabbit assay for BP-55 and BP-75, the specifications of which were described previously [18], and related to morphologic distribution. All samples were assayed in duplicate along with known standards, and chromogenicity was determined by spectrophotometric determination of optical density at the appropriate wavelength using a EAR 400 AT plate reader (SLT Lab Instruments). Standard regression lines, generated by plotting log₁₀ concentration vs log₁₀ optical density, projected correlation coefficients greater than 0.98 in all cases. The maximal allowed sample duplicate error was 10%.

RESULTS

Detection and measurement of cytokines and cytokine inhibitors in malignant ovarian carcinomatous ascites. Cell-free ascites samples derived from 18 patients with histologically proven advanced ovarian carcinoma at the time of original diagnosis were analyzed for the presence of TNF α , LT/TNF β , BP-55 and BP-75, IL-1 β , and IL-1ra by ELISA (Table 1). Both TNF α and LT/TNF β were undetectable, and IL-1 β was present at low levels (mean 0.003 ng/ml). In contrast, BP-55, BP-75, and IL-1ra levels dramatically exceeded those of their

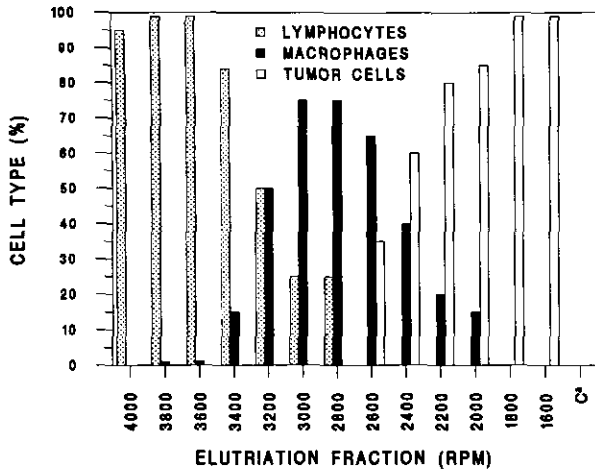


FIG. 1. Elutriation profile for OC1. A single cell suspension derived from OC1 was fractionated by centrifugal elutriation, as described under Materials and Methods. As rotor speed was decreased, cells of progressively increasing size were eluted. The morphology of elutriated cell suspensions was determined by microscopic evaluation of cytopreps stained with Wright-Giemsa and hematoxylin-eosin and is graphed here as the percentage of each major cell type (lymphocytes, macrophages, tumor cells) found in elutriated cell suspensions isolated at specific rotor speeds (rpm).

associated cytokines: BP-55 (mean 2.6 ng/ml), BP-75 (mean 4.3 ng/ml), and IL-1ra (mean 3.3 ng/ml).

Separation and characterization of cell populations derived from solid malignant ovarian carcinoma by centrifugal elutriation. Solid tumor samples, hereafter referred to as OC1-OC3, were obtained from three additional patients with advanced ovarian carcinoma at the time of staging laparotomy. These three patients, ages 51, 67, and 72, were diagnosed with Stage IIIC serous papillary adenocarcinoma of the ovary, grades 3 (OC1 and OC2) and 2 (OC3). Single cell suspensions derived from these solid tumors were then fractionated by centrifugal elutriation, as described under Materials and Methods. Morphology of preelutriated and elutriated cell suspensions was ascertained by microscopic evaluation of cytopreps stained with Wright-Giemsa and hematoxylin-eosin. For all three tumors, morphologic characterization was confirmed independently and in a blinded fashion by a board-certified pathologist. A representative elutriation profile for OC1 is depicted schematically in Fig. 1, and photomicrographs of a hematoxylin-eosin-stained histologic section and cytopreps from preelutriated and most uniform elutriated fractions from the same tumor are presented in Fig. 2. For OC1-OC3, the purity of most homogeneous elutriation fractions ranged from 84 to 99% for lymphocytes, 75 to 92% for macrophages, and 88 to 95% for tumor cells (Table 2).

Spontaneous release of TNF and TNF BP by purified tumor-derived cell populations in short-term cul-

ture. After centrifugal elutriation and morphologic characterization, the purest elutriated cell suspensions with respect to cell type were cultured as described under Materials and Methods, and at predefined incubation times, supernatants were retrieved for analysis by ELISA. Levels of spontaneously released TNF and TNF BP detected in 24-hr culture supernatants are shown in Table 2. For all three tumors, macrophages were the most active in releasing TNF α . Substantial levels of TNF α were also detectable in the tumor cell-rich supernatants. In contrast, TNF α was undetectable in supernatants from all fractions containing greater than 80% lymphocytes. LT/TNF β was undetectable in supernatants from all fractions (data not shown). For all three tumors, at 24 hr, BP-75 was detectable only in the macrophage-rich supernatants. In contrast, BP-55 was detectable in the tumor cell-rich supernatant for OC3 only.

A 48-hr culture time course experiment was designed for OC3 to define the kinetic pattern of spontaneously released TNF and TNF BP. As shown in Fig. 3a, for both macrophage and tumor cell-rich cultures, the TNF α concentration was highest at 6 hr of culture and steadily declined by 48 hr. Throughout the entire 48-hr time course, TNF α was undetectable in supernatants from lymphocyte-rich cultures and, while the data are not shown, LT/TNF β was undetectable in supernatants from all fractions. In contrast, BP-55 was first detectable at 24 hr, in supernatants from the tumor cell-rich culture (Fig. 3b), while BP-75 was detected as early as 6 hr, in supernatants from the macrophage-rich culture (Fig. 3c). At 48 hr, BP-75 was also detectable, albeit at a very low concentration (20 pg/ml), in the supernatant from the tumor cell-rich culture. It is clear that while TNF α levels peaked early in the culture time course and then steadily declined, levels of both inhibitory TNF BP molecules increased to dominate the cultures by 48 hr.

Spontaneous release of IL-1 β and IL-1ra by purified tumor-derived cell populations in short-term culture. The same supernatants from elutriated cell suspensions derived from OC3 also were analyzed by ELISA for the presence of spontaneously released IL-1 β and IL-1ra. As shown in Fig. 4, at 24 hr, very low levels of IL-1 β were released by all cell types. In contrast, IL-1ra was released at high levels, in excess of IL-1 β by approximately 500-fold, in each of these supernatants. Highest levels of both IL-1 β and IL-1ra were found in the supernatant from the macrophage-rich culture, with the supernatants from the tumor cell-rich culture containing intermediate concentrations and lymphocyte-rich culture containing the lowest concentrations. Kinetic studies of IL-1 β and IL-1ra over 48 hr revealed that, for all cell types, IL-1 β was detectable at 6 hr of culture, but concentrations declined (macrophage-rich fraction) or remained constant (lymphocyte

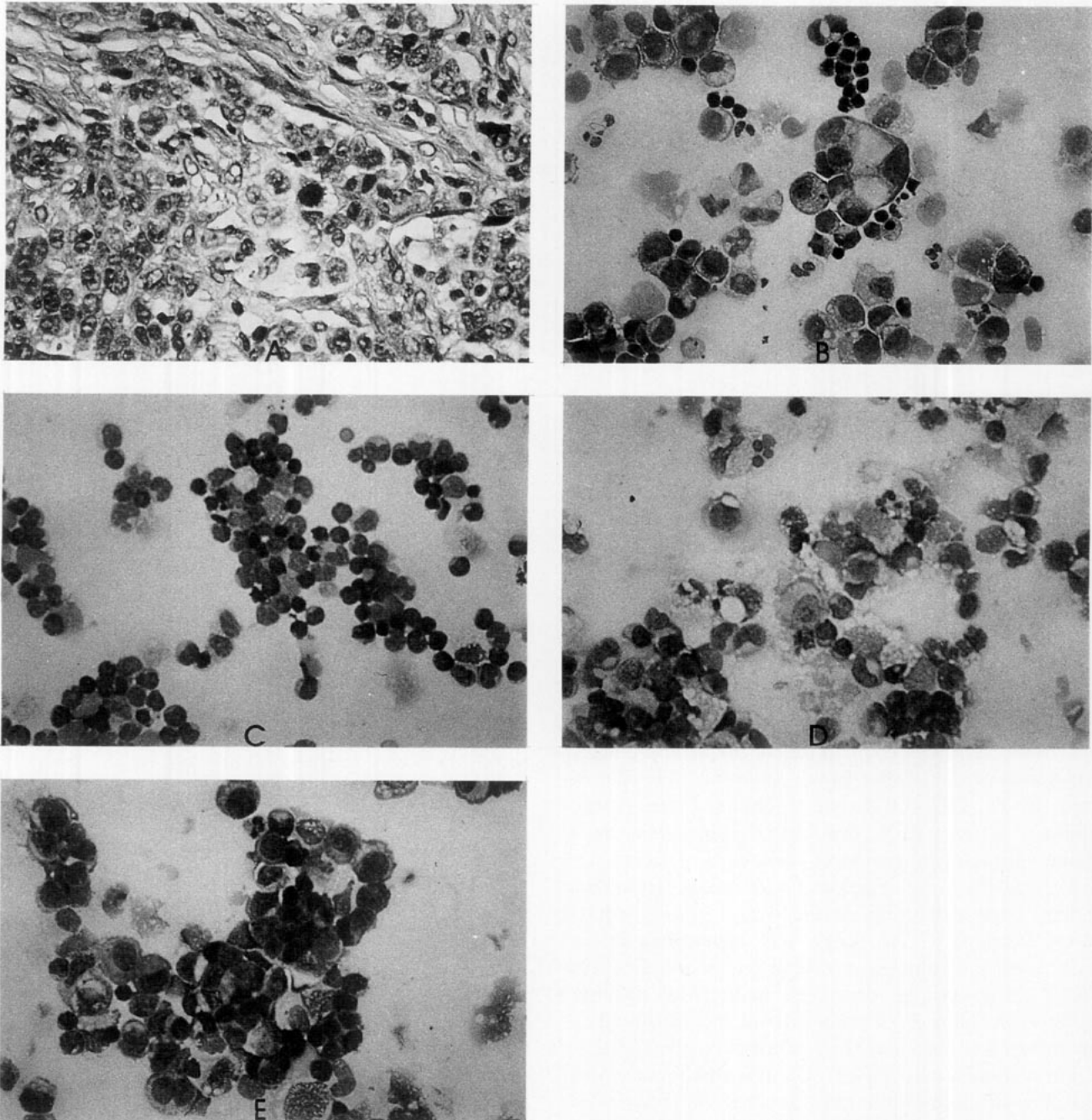


FIG. 2. Morphology of preelutriated and elutriated cell suspensions for OC1 was ascertained by microscopic evaluation of cytopreps stained with Wright-Giemsa and hematoxylin-eosin. Photomicrographs are depicted above. (A) Histologic section of formalin-fixed, paraffin-embedded solid tumor. (B) Praelutriated cell suspension. (C) Lymphocyte-rich cell suspension. (D) Macrophage-rich cell suspension. (E) Tumor cell-rich cell suspension. Original magnifications: 400 \times .

and tumor cell-rich fractions) through 48 hr (Fig. 5a). Figure 5b shows that, for all cell types, IL-1ra was also detectable at 6 hr of culture. In contrast to levels of IL-1 β , which declined or remained constant, however, those of IL-1ra increased in supernatants from all cell types, with levels at 48 hr exceeding 5000 pg/ml for both macrophage and tumor cell supernatants.

DISCUSSION

The cytokines TNF α , LT/TNF β , and IL-1 β were undetectable or present in extremely low levels in the CFA of 18 patients with advanced ovarian carcinoma. In contrast, both species of TNF BP, as well as IL-1ra, were found to be present in great excess over their associated

TABLE 2
Spontaneous Release of TNF α and BP by Purified Tumor-Derived Cell Populations in 24-hr Culture

Cell type	Cytokine/ inhibitor	Tumor		
		OC1	OC2	OC3
Lymphocytes	TNF α (pg/ml)	0	0	0
Macrophages		975	475	220
Tumor cells		71	216	174
Lymphocytes	BP-55 (pg/ml)	0	0	0
Macrophages		0	0	0
Tumor cells		0	0	61
Lymphocytes	BP-75 (pg/ml)	0	0	0
Macrophages		74	562	182
Tumor cells		0	0	0

Note. Cell suspensions from OC1–OC3 were separated by centrifugal elutriation and purest populations of lymphocytes, macrophages, and tumor cells were established in culture. Supernatants from 24-hr cultures were collected, and concentrations of TNF α , BP-55, and BP-75 were determined by ELISA, as described under Materials and Methods.

cytokines in the same ascites samples. The detection of low levels of IL-1 β in our samples is consistent with the findings of other investigators [20]. However, there are conflicting reports in the literature regarding TNF α levels as detected by ELISA in CFA of patients with ovarian carcinoma. Kutteh and Kutteh [20] reported levels of TNF α detected by ELISA in ovarian carcinomatous ascites on the order of 50 pg/ml. In contrast, Wu *et al.* [19] reported TNF α levels in 5 samples ranging from 116 to 1450 pg/ml, with a mean of 444 pg/ml. Because the ELISA we used to measure TNF α employs a neutralizing monoclonal antibody, we can safely conclude that this ELISA would detect only unbound (bioactive) cytokine and that the excess levels of TNF BP we have measured in these samples would prevent detection of bound (bioinactive) TNF α . The apparent discrepancy in TNF α levels in malignant ovarian ascites measured by different investigators [19,20] may reflect (a) differences in the ability of specific monoclonal ELISA systems to detect bound, biologically inactive TNF α , (b) technical variability in background chromogen production, which usually depends on the stringency of ELISA plate washing, or (c) true biologic variability in patient samples. Even if one takes into account this variability, however, the combined mean BP-55 and BP-75 level, as determined in the present study in 18 CFA samples, is at minimum in eightfold molar excess over the maximum level of TNF α detected by Wu *et al.* [19]. In addition, our laboratory previously reported the absence of detectable TNF bioactivity and potent TNF blocking activity in samples of malignant ascites from approximately 20 patients with ovarian ma-

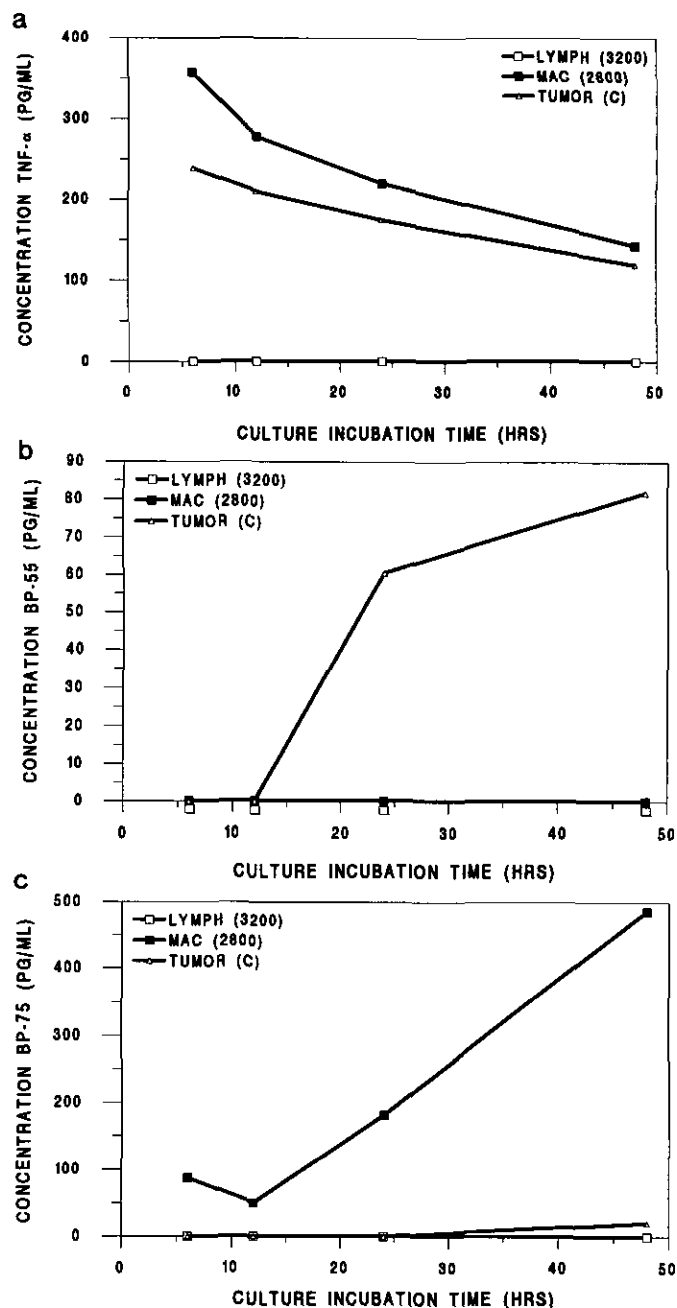


FIG. 3. (a) Kinetics of spontaneously released TNF α in supernatants from lymphocyte-rich (lymph), macrophage-rich (mac), and tumor cell-rich (tumor) cultures derived from OC3 by centrifugal elutriation. Elutriated cell suspensions were cultured for variable incubation times, after which supernatants were obtained for measurement of TNF α by ELISA. (b) Kinetics of spontaneously released BP-55 in supernatants from lymphocyte-rich (lymph), macrophage-rich (mac), and tumor cell-rich (tumor) cultures derived from OC3 by centrifugal elutriation. Elutriated cell suspensions were cultured for variable incubation times, after which supernatants were obtained for measurement of BP-55 by ELISA. (c) Kinetics of spontaneously released BP-75 in supernatants from lymphocyte-rich (lymph), macrophage-rich (mac), and tumor cell-rich (tumor) cultures derived from OC3 by centrifugal elutriation. Elutriated cell suspensions were cultured for variable incubation times, after which supernatants were obtained for measurement of BP-75 by ELISA.

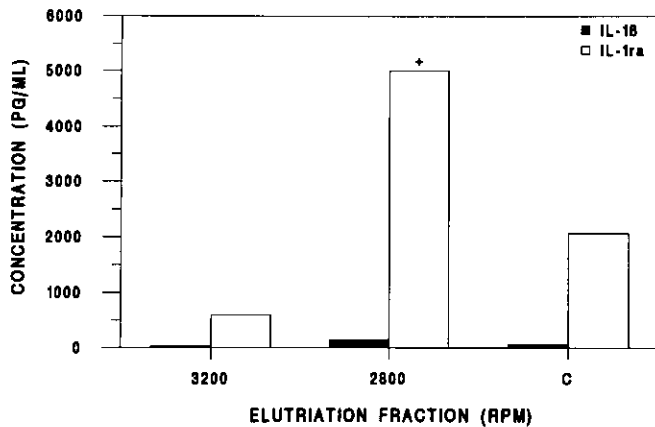


FIG. 4. Levels of spontaneously released IL-1 β and IL-1ra in 24-hr supernatants. Lymphocyte-rich (3200 rpm), macrophage-rich (2800 rpm), and tumor cell-rich (C, Sanderson chamber fraction) cell suspensions derived from OC3 by centrifugal elutriation were cultured for 24 hr, after which supernatants were obtained for measurement of IL-1 β and IL-1ra levels by ELISA. (+ symbolizes levels in excess of 5000 pg/ml.) IL-1 β : 35, 144, and 61 pg/ml for lymphocyte-rich, macrophage-rich, and tumor cell-rich cell suspensions, respectively.

lignancy, as measured by a cytotoxic assay utilizing mouse L929 cells [16,18]. Collectively, these and previous observations suggest that three major mediators of anti-tumor immune responses employed by both lymphocytes and macrophages are blocked in the ascites and serum of women with ovarian carcinoma.

Cytokine and cytokine inhibitors found in ascites can be released by subpopulations of cells within solid tumor. Using a modification of the centrifugal elutriation tech-

nique reported by Moore and Mortari [21], we generated purified suspensions of lymphocytes (84 to 99% pure), macrophages (75 to 92% pure), and tumor cells (88 to 95% pure) from three heterogeneous, solid ovarian adenocarcinomas with yields large enough to facilitate studying cell cultures. There was no loss of cell viability from the elutriation procedure.

Work by other investigators on cytokine expression in cell populations within solid tumor nodules of ovarian carcinomas has been concentrated on assessing mRNA and protein synthesis by *in situ* hybridization and immunohistochemical analysis [22,23]. For example, both TNF α mRNA and protein have been identified within macrophages infiltrating ovarian carcinoma tissue by *in situ* hybridization and immunostaining of fixed sections [22]. We examined the spontaneous release of TNF, IL-1, and their inhibitors in purified, short-term cultures of solid tumor-derived lymphocytes, macrophages, and tumor cells with the hypothesis that our observations would reflect release of these molecules in solid ovarian carcinoma *in vivo*.

Surprisingly, we observed spontaneous release of both TNF α and IL-1 β from cultures of tumor-associated macrophages. This was an unexpected finding, since there is little or no TNF and IL-1 in ascites and little TNF α detected by immunohistochemical analysis of macrophages infiltrating solid tumors [22]. The finding that cytokine release was spontaneous rather than artifactually induced by enzymatic tumor dissociation is supported by the observation that concentrations of digestive enzymes used for tumor disaggregation did not stimulate the release of

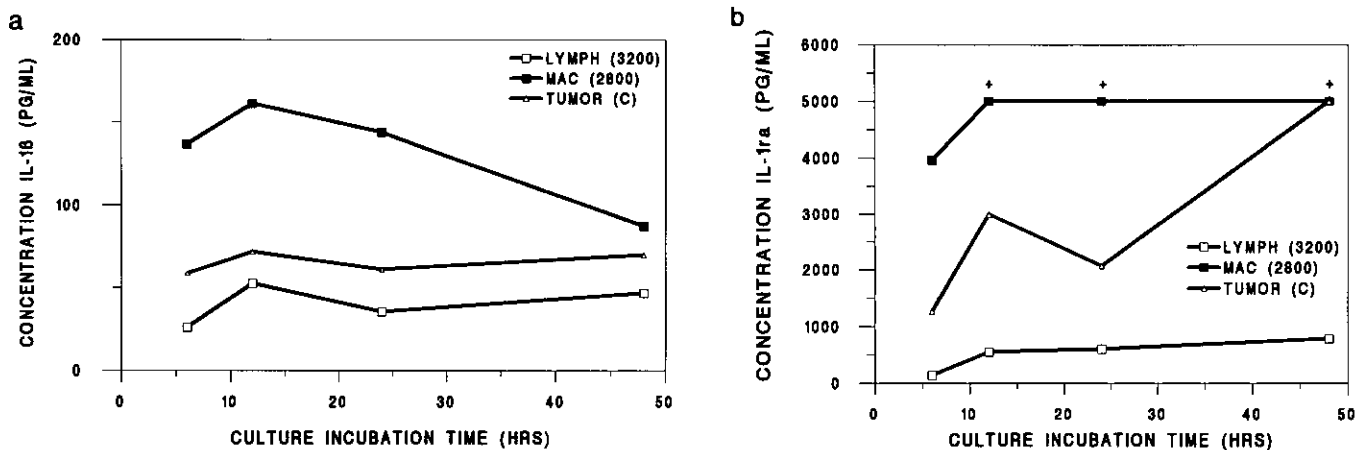


FIG. 5. (a) Kinetics of spontaneously released IL-1 β in supernatants from lymphocyte-rich (lymph), macrophage-rich (mac), and tumor cell-rich (tumor) cultures derived from OC3 by centrifugal elutriation. Elutriated cell suspensions were cultured for variable incubation times, after which supernatants were obtained for measurement of IL-1 β by ELISA. (b) Kinetics of spontaneously released IL-1ra in supernatants from lymphocyte-rich (lymph), macrophage-rich (mac), and tumor cell-rich (tumor) cultures derived from OC3 by centrifugal elutriation. Elutriated cell suspensions were cultured for variable incubation times, after which supernatants were obtained for measurement of IL-1ra by ELISA. (+ symbolizes levels in excess of 5000 pg/ml.)

TNF α from THP-1 or HL-60 human monocytic cell lines (data not shown). Since TNF α and IL-1 β are inducible and macrophages do not normally release these cytokines without stimulation [24–27], these results indicate that macrophages are present in an “activated” state within solid ovarian carcinoma. However, they are apparently not releasing TNF α *in vivo*. Erroi *et al.* [28] measured IL-1 release by solid ovarian carcinoma-associated macrophages isolated by plastic adherence with a thymocyte costimulator (biologic) assay and found “no appreciable levels of IL-1 spontaneously” released in 24-hr culture. This bioassay result is not inconsistent with our data. Although we demonstrated spontaneous release of IL-1 β by cultured, tumor-derived macrophages, the relatively high levels of IL-1ra released in the same culture could well account for the inhibition of IL-1 bioactivity observed previously.

Ovarian carcinoma cells also released a small amount of TNF α and IL-1 β . However, we cannot exclude the possibility that the majority of TNF α and IL-1 β might have been released by the small number of macrophages which contaminated these cultures. Nevertheless, the low-level spontaneous release of TNF α and IL-1 β from ovarian tumor cells is consistent with the results of recent studies from other laboratories [19,22,23,29].

Although many studies have demonstrated that tumor-derived lymphocytes can be activated *ex vivo* with cytokines and other biological response modifiers to exert cytolytic activity against autologous and heterologous ovarian cancer cells [5], the absence of TNF α or TNF β in supernatants from tumor-associated lymphocyte-rich cell suspensions suggests that lymphocytes infiltrating ovarian carcinoma are inactive at the tumor site.

The most significant finding in the present study was that both tumor cells and tumor-associated macrophages from solid ovarian carcinoma tissue spontaneously release inhibitors for TNF and IL-1. BP-75 was released primarily by tumor-associated macrophages and BP-55 by tumor cells. Consistent with these findings are recent independent data demonstrating that the 55-kDa TNF membrane receptor is the predominant form expressed on ovarian carcinoma cells and that the 75-kDa TNF membrane receptor is the predominant form expressed on infiltrating macrophages by *in situ* hybridization and immunostaining of fixed sections [22]. Unfortunately, in the latter study, no distinction could be made between the released and cell-associated forms of these molecules. Because the supernatant from only one 1 of 3 tumors in the present study contained BP-55 and because levels of BP-55 on the order of 2 ng/ml have been identified in carcinomatous ascites and in the sera from women with this disease, this molecule may originate *in vivo* from sources other than tumor. It is clear that TNF BP released

in these cultures could form complexes with TNF, thereby not only blocking bioactivity but also masking antigenic sites on the TNF molecule. Similarly, the relatively low levels of IL-1 β released by ovarian carcinoma cell lines (compared to normal ovarian epithelial cells) demonstrated in a recent study by Ziltener *et al.* [29] may reflect autocrine or paracrine downregulation by inhibitors such as IL-1ra, which we have found released in relative abundance by tumor cells as well as tumor-associated macrophages. At present, there are no reported data from other groups on IL-1ra in this disease. Future investigation will specifically probe which mechanisms regulate the release of TNF and IL-1 inhibitors by purified cell populations derived from solid ovarian carcinomas.

The roles of TNF and IL-1 in the ovarian carcinoma disease process have yet to be clearly defined. An experimental xenograft model in nude mice suggests a direct therapeutic effect of intraperitoneal TNF α administration *in vivo* [30]. TNF α has demonstrated direct cytotoxic activity against ovarian carcinoma cells freshly isolated from solid tumor *in vitro* [31]. Recent *in vitro* work has suggested that local levels of TNF α may function as an autocrine/paracrine growth factor in this disease and that IL-1 may be a cofactor in this process [19,32,33]. The observation by Wu *et al.* [19] that high local levels of TNF α , on the order of 1 to 10 ng/ml, can act as a growth factor for ovarian carcinoma cell lines *in vitro* raises some concern that this molecule may promote the pathogenesis of this disease. However, Wu *et al.* did show that the growth factor effect of IL-1 could be blocked by exogenous recombinant TNF BP. Therefore, the excess of TNF BP and IL-1ra in the local tumor milieu and in ascites is likely to suppress these and the more well-established antitumor properties of TNF and IL-1, such as direct cytokine-mediated cytotoxicity *in vitro* and *in vivo* as well as recruitment of host effector mechanisms [9,10].

Our data at present lend support to the notion that the local and regional environment of ovarian carcinoma *in vivo* is antagonistic to TNF and IL-1 function. The uncontrolled production of TNF and IL-1 inhibitors by cells within solid tumor and subsequent accumulation of these molecules in ascites might explain the disappointing results of regional cytokine therapy trials [5]. Whether this phenomenon contributes to the pathogenesis of this disease remains to be determined.

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