Inhibition of Human Lymphoblastoid Cell Line Proliferation by Ascites Fluids from Ovarian Cancer Patients

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ABSTRACT

Ascitic fluids from ovarian cancer patients which contained a component (lymphocyte-inhibitory activity) that inhibited the blastogenic responses of normal lymphocytes were tested for their effect on established lymphoblastoid cell lines. These ascites fluids inhibited proliferation as measured by [3H]thymidine uptake and cell growth in vitro of two different B- (SB and IM-1) and two different T- (HSB and CEM) lymphoblastoid cell lines but were not cytotoxic. No difference in the sensitivity of T- and B-lymphoblastoid cell lines to the active component in the ascites fluids could be demonstrated. In contrast, established nonlymphoid cell lines (HEP-2, oligodendrogloma, and bladder tumor cell lines) were unaffected by the ascites fluids. Two different preparations of partially purified lymphocyte inhibitory activity from these ascites fluids also inhibited lymphoblastoid cell line proliferation. In addition, both B- and T-lymphoblastoid cell lines absorbed and/or metabolized the lymphocyte-inhibitory activity at 37°C but not at 4°C. These data suggest that the inhibition of lymphoblastoid cell line proliferation and the inhibition of the blastogenic responses of normal lymphocytes by the ascites fluids are attributable to the same or similar factors.

INTRODUCTION

Advanced neoplastic disease has often been associated with a compromised state of immune function (10, 12, 23). At present, the mechanisms accounting for depressed in vitro and in vivo lymphocyte responsiveness remain unclear. Recent studies have demonstrated the presence of serum factors in patients with advanced cancer which nonspecifically modulate lymphocyte function in vitro and in vivo (9, 17, 22, 25, 27, 30, 35). Similar inhibitory factors have been found in the serum of normal individuals in a variety of species (1, 5-7, 11, 24, 36). Occchino et al. (28) have described an immunoregulatory peptide that is noncovalently linked to an a-globulin which is found in low quantities in NHS.3 Recent findings suggest that this peptide may regulate T-lymphocyte function in vitro and in vivo (7, 8, 28). Similarly, Hanna et al. (13) and Nelken et al. (25) have identified an immunosuppressive protein which inhibits both B- and T-lymphocyte function. Several other serum- and cell-associated components have been reported to modulate lymphocyte responses and are thought to play a role in the regulation of the immune response in vivo (29, 34). It has been postulated that high levels of these serum immunoregulatory factors which nonspecifically modulate lymphocyte responsiveness may account for the depressed immune status of anergic cancer patients (9, 12, 27).

Recent studies have demonstrated the presence of soluble immunosuppressive factors in cell-free ascites and pleural fluids in patients with peritoneal metastatic disease and in ascites fluids from murine tumor models (2-4, 14, 16, 21, 31-33, 36). The presence of these factors in fluids containing tumor cells has been postulated to effect local immunosuppression (3, 4). Hess et al. (14, 15) have demonstrated that a substantial number of ascites fluids from ovarian cancer patients contain a nondialyzable, noncytotoxic factor(s) which is capable of inhibiting the in vitro response of normal lymphocytes. The active factor(s) in these fluids was found to have an apparent molecular weight between 40,000 and 80,000 and migrated with the albumins upon preparative electrophoresis (15). In addition, preliminary studies revealed that these fluids were capable of inhibiting proliferation of lymphoblastoid cells but were found not to have an effect on other established cell lines, suggesting that there was a selective effect on cells of lymphoid origin. The present investigations were undertaken to characterize the inhibition of lymphoblastoid cell line proliferation by ascites fluids from ovarian cancer patients and to determine if this inhibitory activity is attributable to the active component which inhibits the proliferative response of normal lymphocytes.

MATERIALS AND METHODS

Preparation of Samples. Ascites fluids were obtained during surgery at Duke University Medical Center from patients suspected of having ovarian cancer. Fluids were clarified by centrifugation (1800 x g; 30 min), aliquoted, and stored at -20°C as described previously (14). Prior to testing, all samples were sterile filtered through a Millex 0.22-µm filter (Millipore Corp., Bedford, Mass.).

Purification of Lymphocyte-Inhibitory Activity. The partial purification of the active component from active ascites fluids which accounted for inhibition of in vitro lymphocyte function has been described in detail elsewhere (15). Briefly, ascites fluids containing lymphocyte-inhibitory activity were chromatographed over Con A-Sepharose 4B. The material that did not bind to the column which contained the majority of the inhibitory activity was then chromatographed over DE-52 cellulose (column equilibrated with 0.03 M NaCl:0.01 M potassium phosphate buffer). The absorbed material which contained the inhibitory activity was eluted with 1.0 M NaCl:0.01 M potassium phosphate buffer.

1 This investigation was supported in part by American Cancer Society Grant IM-04, the Ettie Stiehleimer Memorial Fund, and NIH Grant CA-14049.
2 NIH Postdoctoral Fellow on Training Grant T32 CA-09058-03. Present address: Oncology Center 3-130, Johns Hopkins Hospital, Baltimore, Md. 21205. To whom requests for reprints should be addressed.
3 The abbreviations used are: NHS, normal human serum; Con A, concanavalin A; MLC, mixed-lymphocyte culture; PHA, phytohemagglutinin; Dulbecco’s PBS, Dulbecco’s phosphate-buffered saline (Grand Island Biological Co., Grand Island, N. Y.).

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phosphate buffer at pH 7.0. The active fraction was then subjected to preparative electrophoresis in 6.5% polyacrylamide gels. The active moiety resided in the fraction which migrated with albumins. Prior to testing for activity, the fractions were buffer exchanged either by chromatography over Sephadex-G-25 or by ultrafiltration on UM-10 membranes (Amicon Corp., Lexington, Mass.) using 0.15 M NaCl:0.01 M potassium phosphate (pH 7.0) as the exchange buffer. The samples were then concentrated on UM-10 membranes and sterile filtered through a Millex 0.22-µm filter.

General Tissue Culture Methods. The proliferative response of normal human peripheral blood lymphocytes to mitogenic challenge or in MLC was determined as described previously (14). Briefly, normal human peripheral blood lymphocytes isolated by density centrifugation on a gradient of lymphocyte separation medium (Litton Bionetics, Kensington, Md.) were washed and resuspended in complete medium containing Roswell Park Memorial Institute Tissue Culture Medium 1640, 15% pooled NHS supplemented with 1% L-glutamine, penicillin (150 units/ml), and streptomycin (150 µg/ml). Microcultures were established in flat-bottomed microtiter plates (No. 76-003-05; Linbro Chemical Co., New Haven, Conn.) by adding 0.1 ml of the cell suspension (2.0 x 10⁶ lymphocytes) and 0.1 ml of complete medium to each well. Doses of PHA (reagent grade; Burroughs Wellcome Co. and Co., Research Triangle Park, N. C.) or Con A (Pharmacia Fine Chemicals, Uppsal, Sweden) which had been previously determined to be optimal in our laboratory were used for mitogen stimulation (0.05 ml/well). Bidirectional MLC’s were established by adding 1.5 x 10⁶ lymphocytes from 2 unrelated individuals to wells of the microtiter plate in a final volume of 0.25 ml. All tests were done in triplicate, and the cultures were incubated at 37° in a humidified 5% CO₂ incubator. On Day 3 of the PHA cultures, Day 4 of the Con A cultures, and Day 6 of the MLC cultures, 1 µCi of [³H]thymidine (specific activity, 1.9 Ci/mmol; Schwarz/Mann, Orangeburg, N. Y.) was added to each well prior to harvesting on glass-fiber filter paper (Grade 934 AH; H. Reeve Angel & Co., Clifton, N. J.) with a multiple automated sample harvester. The filter paper was dried and processed for liquid scintillation counting.

Several inhibitory ascites fluids were tested for their effect on growth of established cell lines maintained in the Division of Immunology at Duke University. Cell lines that were tested included HSB and CEM, T-lymphoblastoid cell lines; SB and IM-1, B-lymphoblastoid cell lines; HeP-2, a human epithelioma; an oligodendroglioma cell line; and a bladder tumor cell line. The lymphoblastoid cell lines grow as a single-cell suspension and were harvested by centrifugation. The HeP-2, oligodendroglioma, and bladder tumor cell lines were processed by trypsinization (0.25%) (19). The cells were washed 3 times with Roswell Park Memorial Institute Tissue Culture Medium 1640 and resuspended in complete medium as described above. Cells were plated at a concentration of 1 x 10⁶ cells/well. The cultures were incubated for 1 to 2 days, at which time the cultures were labeled with 1 µCi of [³H]thymidine, incubated for 16 to 18 hr, and harvested in a fashion similar to the lymphocyte cultures. Cultures were also enumerated and checked for viability by trypan blue type exclusion and phase-contrast microscopy at the end of the incubation period.

Testing of Ascitic Fluids and Purified Samples for Inhibitory Activity. Several ascites fluids and purified fractions were tested for inhibitory activity by adding 40 µl of the test fluids to triplicate wells at the initiation of culture. Control responses represent additional triplicate tests to which 40 µl of NHS, 40 µl of Dulbecco’s PBS, and/or 40 µl of complete medium were added. Titration of the inhibitory activity was accomplished by adding graded amounts of the highly active whole ascites fluids (40, 30, 20, and 10 µl/well) at the initiation of the culture with the volumes being equalized by addition of Dulbecco’s PBS (final culture volume, 0.29 ml).

Results are expressed as the mean cpm of [³H]thymidine uptake ± S.D. In some experiments, for the sake of comparison, the percentage of inhibition was calculated according to the following formula:

\[
\text{mean cpm of response in test fluid} = \frac{\text{average mean cpm of NHS, Dulbecco’s PBS and complete medium controls}}{100}
\]

Data were analyzed for statistical significance by using Student’s t test.

RESULTS

Inhibition of Lymphoblastoid Cell Proliferation and the Blastogenic Response of Normal Lymphocytes. Ascites fluids from patients with malignant and benign ovarian disease were tested for their ability to inhibit the in vitro blastogenic response of normal lymphocytes, and the results have been described in detail elsewhere (14). In brief, 12 of 26 ascites fluids were found to contain a noncytotoxic factor(s) which inhibited mitogen and MLC responses of normal lymphocytes. Eleven of the fluids considered positive were from patients with various classes of malignant ovarian neoplasms (24 ovarian cancer patients tested). The other positive fluid found to contain the lymphocyte-inhibitory activity was the pelvic fluid (arbitrarily classified as an ascites fluid) from a patient with a benign follicular cyst. The remaining ascites fluids (14) from both benign and malignant lesions did not alter the in vitro lymphocyte response. Of the 12 positive ascites fluids, 6 (5 from patients with malignant ovarian neoplastic disease and the fluid from the patient with the benign follicular cyst) were tested for their ability to alter lymphoblastoid cell line proliferation.

The inhibitory titer of a representative ascites fluid from a patient with ovarian adenocarcinoma found to contain the active component in the PHA, Con A, and MLC assays is illustrated in Chart 1A. All 3 assays of lymphocyte responsiveness were inhibited when the active ascites fluid (40, 30, and 20 µl) was added to the cultures. Minimal or no inhibition of the lymphocyte response in the mitogen assays was observed when 10 µl of the fluid were added to the cultures. At this dose, the MLC response of normal lymphocytes was significantly (p < 0.001) inhibited, indicating that the MLC response was more sensitive to the effect of the inhibitory component in the ascites fluid than were the mitogen responses. Chart 1B illustrates a representative experiment showing that addition of this active ascites fluid to cultures of the lymphoblastoid cells resulted in significant (p < 0.001) inhibition of proliferation as measured by [³H]thymidine uptake. Proliferation of both B- (SB, IM-1) and T- (HSB, CEM) lymphoblastoid cell lines was similarly inhibited by this active fluid. Moreover, the titration of the inhibitory activity against the lymphoblastoid cell lines was similar to that observed for the inhibition of the response of peripheral blood lymphocytes. No consistent difference could

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be demonstrated between the inhibitory titer for B- and T-lymphoblastoid cell lines. In contrast to the inhibition of the lymphoid cells, no significant inhibition of proliferation as measured by [3H]thymidine uptake could be demonstrated for the nonlymphoid cell lines (HEp-2, oligodendroglioma, and bladder tumor) when they were cultured in the presence of this ascites fluid containing the lymphocyte-inhibitory activity. Cells were tested for viability by trypan blue dye exclusion after 24 and 48 hr of incubation, and the results (data not shown) demonstrated that the fluids were not cytotoxic, a finding previously tested for viability by trypan blue dye exclusion after 24 and fluid containing the lymphocyte-inhibitory activity. Cells were thymidine uptake of HSB and SB cell lines. The results are shown for peripheral blood lymphocytes using both trypan blue dye exclusion and a 51Cr-labeled release assay (14).

**Table 1**

<table>
<thead>
<tr>
<th>Ascites fluid</th>
<th>[H]Thymidine uptake (cpm)</th>
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<tbody>
<tr>
<td></td>
<td>Response in presence of 40 µl of ascites fluids/well</td>
</tr>
<tr>
<td>489.</td>
<td>797 ± 797 (97.8)</td>
</tr>
<tr>
<td>501.</td>
<td>3,975 ± 2,216 (89.0)</td>
</tr>
<tr>
<td>533.</td>
<td>129 ± 15 (99.8)</td>
</tr>
<tr>
<td>534.</td>
<td>125 ± 61 (99.8)</td>
</tr>
<tr>
<td>503.</td>
<td>3,614 ± 1,224 (97.6)</td>
</tr>
<tr>
<td>503. Purified Preparation 1c</td>
<td>3,029 ± 244 (96.2)</td>
</tr>
<tr>
<td>503. Purified Preparation 2d</td>
<td>1,873 ± 766 (97.6)</td>
</tr>
<tr>
<td>501.</td>
<td>1,673 ± 6,664 (94.2)</td>
</tr>
<tr>
<td>506.</td>
<td>1,445 ± 197 (96.2)</td>
</tr>
<tr>
<td>506. Purified Preparation 2d</td>
<td>1,772 ± 126 (97.8)</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* Numbers in parentheses, percentage of inhibition based on comparison to control response.

This is followed by preparative electrophoretic purification. Protein concentrations of Samples 503 and 526 were 25.8 and 27.3 mg/ml, respectively.

A variety of highly active lymphocyte-inhibitory fluids and 2 different purified preparations of the active component from 2 different ascites fluids also resulted in significant (p < 0.001) inhibition of the proliferative response of both HSB and SB cells as defined by [3H]thymidine uptake. Similar results were obtained when the other 2 lymphoblastoid cell lines (CEM and IM-1) were cultured with the active fluids. In contrast, several ascites fluids not found to contain lymphocyte-inhibitory activity as determined by their inability to inhibit the normal lymphocyte PHA and MLC blastogenic responses when tested.

Inhibition of Human Lymphoblastoid Cell Line Proliferation

Chart A, titration of an inhibitory ascites fluid in the PHA, Con A, and MLC response assays of normal human peripheral blood lymphocytes. The data are presented as the percentage of inhibition based on the mean control response of assays with 40 µl of NHS, 40 µl of Dulbecco's PBS, and/or 40 µl of complete medium added to the culture. The control responses for the PHA, Con A, and MLC assays in cpm of [3H]thymidine uptake were: 58,720 ± 3,703 (S.D.); 129,178 ± 6,163; and 19,231 ± 677; respectively. Values of [3H]thymidine uptake for unstimulated lymphocytes never reached above 1500 cpm. B, titration of an inhibitory ascites fluid against several different established cell lines. The data are presented as the percentage of inhibition compared to control assays with 40 µl of NHS, 40 µl of Dulbecco's PBS, and/or 40 µl of complete medium added to the culture system. The control responses for each established cell line in cpm of [3H]thymidine uptake were: SB, 76,478 ± 3,258; HSB, 64,106 ± 641; CEM, 262,028 ± 3,851; IM-1, 115,394 ± 6,592; HEp-2, 106,513 ± 3,534; oligodendroglioma, 254,611 ± 17,859; and the bladder tumor cell line, 60,274 ± 7.526.
Inhibition of proliferation and \([1^H]\text{thymidine uptake by an ascitic fluid containing lymphocyte-inhibitory activity}\)

Table 2

<table>
<thead>
<tr>
<th>Ascitic fluid added to culture wells</th>
<th>([1^H]\text{Thymidine uptake (cpm)})</th>
<th>Viable cell recovery (total no. of viable cells x 10^5/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSB</td>
<td>SB</td>
<td>HSB</td>
</tr>
<tr>
<td>40 µl</td>
<td>111 ± 36a,b</td>
<td>125 ± 61b</td>
</tr>
<tr>
<td>10 µl</td>
<td>10,103 ± 265c</td>
<td>80,215 ± 942c</td>
</tr>
<tr>
<td>NHS added to culture wells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 µl</td>
<td>8,999 ± 226</td>
<td>76,048 ± 738</td>
</tr>
<tr>
<td>10 µl</td>
<td>10,176 ± 114</td>
<td>79,751 ± 1,617</td>
</tr>
</tbody>
</table>

\(a\) Mean ± S.D.

\(b\) Significantly \((p < 0.001)\) different compared to control response in NHS.

\(c\) Not significant compared to control response.

As judged by the number of cells harvested from culture. In contrast, the number of cells recovered from culture with 40 µl of the inhibitory ascites fluid was not significantly different from the number of cells plated, indicating no net proliferative growth. At the noninhibitory dose of ascites fluid (10 µl/well), no significant difference in \([1^H]\text{thymidine uptake}\) and viable cell recovery could be demonstrated compared to the NHS (10 µl) control cultures. The data indicate that proliferation as measured by \([1^H]\text{thymidine uptake}\) as well as replication was inhibited by the ascites fluids containing lymphocyte-inhibitory activity.

Absorption of Lymphocyte-inhibitory Activity by Lymphoblastoid Cell Lines. To determine whether preincubation of HSB and SB cells removed the lymphocyte-inhibitory activity from the active fluids, 1.5 x 10^5 HSB and/or SB cells were incubated with 0.5 ml of inhibitory ascites fluids at 4°C and 37°C. After 24 hr of incubation, the suspension of cells and ascites fluids was centrifuged at 500 x g for 30 min. The supernatant was then tested for its ability to inhibit freshly isolated lymphocytes in a PHA assay; results of a representative experiment are shown in Chart 2. Incubation of both HSB and SB cells with an active ascites fluid (526) at 4°C showed no substantial loss of inhibitory activity by absorption. In contrast, the active ascites fluid incubated with both the B- and T-lymphoblastoid cell lines at 37°C resulted in a significant (HSB: 40 µl, \(p < 0.01\); 30 µl, \(p < 0.001\); 20 µl, \(p < 0.05\); SB: 40 µl, \(p < 0.001\); 30 µl, \(p < 0.001\); 20 µl, \(p < 0.05\)) absorption of the active moiety which inhibits the PHA response of normal lymphocytes.
loid cell lines but were not cytotoxic. No consistent difference in the sensitivity of the B- and T-lymphoblastoid cell lines could be documented by titration of the ascites fluids which contained the inhibitory activity into the culture. Moreover, similar levels of inhibition were observed as judged by titration of the ascites fluids into culture, when the active component(s) were tested for their ability to inhibit lymphoblastoid cell line proliferation and for their ability to inhibit the blastogenic responses of normal lymphocytes. In addition, the partially purified lymphocyte-inhibitory activity which inhibited the proliferative response of normal lymphocytes (14) was also found to effectively inhibit lymphoblastoid cell line proliferation, suggesting that both activities were the result of the action of the same or similar components. This concept was further strengthened by the fact that both the B- and T-lymphoblastoid cell lines absorbed the component of ascites fluid which inhibited the lymphocyte responses. The absorption and/or removal of the active component by the lymphoblastoid cell lines was found to be temperature dependent, suggesting that metabolically active cells were required to absorb and/or metabolize the active moiety, a finding similarly ascribed to the absorption of the inhibitory factor by normal human peripheral blood lymphocytes (14). The active moiety contained in the ascites fluids which was responsible for the inhibition of in vitro lymphocyte function has been partially purified and characterized (15). The inhibitory factor was found to have a molecular weight between 40,000 and 80,000 and migrated with the albumins upon preparative electrophoresis. A small molecular component with inhibitory activity could not be separated as was demonstrated for other immunoregulatory molecules isolated from human serum (8, 28).

Recent studies have suggested that the inhibition of [3H]-thymidine uptake in malignant lymphoid cell lines by an inhibitor such as a lymphocyte chalone may not be associated with a decrease in their rate of cell division (29). In our studies, the ascites fluids containing lymphocyte-inhibitory activity not only inhibited [3H]-thymidine uptake but also resulted in no net proliferative growth as judged by the fact that the number of cells recovered from culture with an inhibitory dose of ascites fluid was not significantly different from the number of cells initially cultured. In contrast, control cultures and cultures with noninhibitory doses of the ascites fluids demonstrated approximately a 2-fold expansion of the number of cells in culture. These results strongly suggest that replication and growth as measured by an increase in cell numbers in the culture system were effectively inhibited by these ascites fluids.

Although proliferation of both B- and T-lymphoblastoid cell lines was effectively inhibited by the ascites fluids, established nonlymphoid cell lines were unaffected by these fluids, suggesting that there may be a selective effect on lymphoid cells. However, the effect of the inhibitory ascites fluids on proliferation of many other established cell lines of various origins must be tested in order to substantiate any selective effect of the inhibitor on lymphoid cells. In agreement with our findings are the studies of Badger et al. (3), who demonstrated that immunosuppressive ascites fluids from patients with peritoneal metastatic cancer failed to inhibit proliferation of HeLa cells. Similar results were obtained by Nelken et al. (26), who showed that normal immunosuppressive protein isolated from NHS effectively inhibited B- and T-lymphoblastoid cell proliferation but did not affect proliferation of myeloid cells and fibroblasts.

It is presently unknown if these inhibitors have a selective effect on cells of lymphoid origin via a specific receptor for the inhibitory molecule present on lymphoid cells or if this selective effect reflects a differential sensitivity to the inhibitor on a cellular or metabolic basis with lymphoid cells being most sensitive to the effects of the inhibitory molecule. It may be that cells of nonlymphoid origin such as the ones used in the present studies require extremely high levels of the inhibitory component before inhibition of proliferation occurs.

The origin of the inhibitory factor which inhibits lymphoblastoid cell line proliferation and normal lymphocyte blastogenesis found in ascites fluids from ovarian cancer patients is currently unknown. It is possible that the tumor cells produce or induce the formation of this inhibitory factor. In our previous investigations, the occurrence of lymphocyte-inhibitory activity was not limited to effusions from malignant tumors but was found in 2 fluids originating from benign conditions (14), a finding similar to that described by Badger et al. (2). In their studies, ascites fluids from patients with cancer metastatic to the peritoneum as well as ascites fluids from patients with liver disease such as cirrhosis contained the inhibitory activity. The presence of lymphocyte antiproliferative factors in ascites fluids may constitute a natural host-mediated response to inflammatory stimuli which is not limited to malignant neoplasia (14, 18). However, the frequency of ascitic fluids containing lymphocyte-inhibitory activity in cancer patients (2-4, 14) and in several murine tumor models (16, 21, 31-33, 36) would indicate that malignant neoplasia is often associated with an excess production of immunosuppressive factors. High levels of a lymphocyte-inhibitory factor(s) such as the one described in the present studies, whether produced or induced by tumor cells, may lead to the general impairment of immune responsiveness that is often associated with advanced neoplastic disease (10, 12, 20, 23).

In summary, ascites fluids containing lymphocyte-inhibitory activity from ovarian cancer patients effectively suppressed proliferation of both B- and T-lymphoblastoid cell lines but did not affect established cell lines of nonlymphoid origin. The inhibitory activity in these fluids responsible for the inhibition of lymphoblastoid cell proliferation as well as the inhibition of the blastogenic responses of normal lymphocytes appeared to be attributable to the same or similar components. The action of this inhibitory component on the lymphoblastoid cell may be an excellent model system to further study the regulation of lymphoid cell proliferation and to further characterize the mode and mechanism of action of the lymphocyte-inhibitory activity associated with the ascitic fluids from ovarian cancer patients.

REFERENCES

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