Inhibition of in Vitro Lymphocyte Function by Cystic and Ascitic Fluids from Ovarian Cancer Patients

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ABSTRACT

Ascitic and cystic effusions from patients with benign and malignant ovarian neoplasms were tested for the presence of immunosuppressive activity as measured by the inhibition of in vitro lymphocyte function. A substantial percentage of ascites fluids (12 of 26) and a few (2 of 11) cystic effusions from primary ovarian tumors were found to contain a factor(s) which was capable of inhibiting the blastogenic response of normal lymphocytes. The presence of lymphocyte-inhibitory activity was not limited to fluids derived from malignant neoplastic processes but was also found in effusions associated with benign ovarian disorders. Inhibition of the lymphocyte response by active ascites fluids inversely correlated with the cell and serum concentrations used in the assay system. Absorption of the active moiety from the inhibitory ascites fluids by normal lymphocytes was found to be time and temperature dependent and did not appear to be readily reversible. The factor(s) responsible for the inhibition of lymphocyte function was non-dialyzable, was nontoxic, and had an apparent molecular size between 50,000 and 100,000 as estimated by Diaflo ultrafiltration. The inhibitory activity was found to be moderately sensitive to extreme acid and alkaline conditions and was stable to a 56°, 30-min heat treatment. Preliminary results revealed that the ascites fluids containing lymphocyte-inhibitory activity inhibited the proliferation of both T- and B-lymphoblastoid cell lines as measured by [3H]thymidine incorporation. However, no effect was observed on the proliferation of epithelioma and oligodendroglioma cell lines.

INTRODUCTION

Advanced neoplastic disease has often been associated with a general impairment of immune responsiveness (10, 11, 21). However, the mechanisms which account for depressed lymphocyte function remain unclear. Recent studies (9, 15, 20, 24, 25, 28, 34) have demonstrated the presence of factors in the serum of patients with advanced cancer, which nonspecifically inhibit in vitro and in vivo lymphocyte function. Similar inhibitory factors have been found in serum from normal individuals, although in much lower quantity, and are associated with the α-globulins (7, 12, 22, 26, 27, 33). More recent studies have shown the presence of soluble immunosuppressive factors in cell-free AF's3 from patients with peritoneal metastatic disease (3, 4, 31) and in ascites from several murine tumor models (14, 19, 30—32, 36). These factors are similar in their range of activity to those described in serum and could account for the depressed immune status of these patients (1, 3, 30, 31).

Studies by Badger et al. (3) have shown that high levels of lymphocyte-inhibitory activity could be found in AF's in the absence of any serum immunosuppressive activity. On this basis, they postulated that tumor cells may locally produce or induce the formation of factors capable of inhibiting lymphocyte function, thus effecting local immunosuppression. It follows that immunosuppressive activity should be frequently found in ascites and in other effusions of neoplastic origin. The present investigations were undertaken to examine the immunosuppressive activity in cystic and ascitic fluids associated with ovarian neoplasms and to determine its range and mode of action.

MATERIALS AND METHODS

Preparation of Samples. Ascitic fluids and cystic effusions were obtained aseptically during surgery at Duke University Medical Center from patients suspected of having ovarian cancer. Serum samples were obtained at the same time whenever possible. These patients were not on cytotoxic drug chemotherapy or radiation therapy at the time the samples were taken. The samples were centrifuged at 1800 × g for 30 min to remove cellular debris, numerically coded, aliquoted, and stored at −20°. Prior to testing, all samples were sterile filtered through a Millex 0.22-μm filter (Millipore Corp., Bedford, Mass.). Protein concentrations of AF's and cystic effusions ranged from 10 to 50 mg/ml as estimated spectrophotometrically by absorbance at 280 nm. The mean protein concentration for positive (containing lymphocyte-inhibitory activity) and negative fluids were 32.8 ± 13.1 and 44.5 ± 9.7 (S.D.) mg/ml, respectively. There was no relationship between the initial protein concentration and the presence of lymphocyte-inhibitory activity in original ascites and cystic fluids.

Heat sensitivity of the suppressive activity was tested by heating samples to 56, 62, or 100° for 30 min. Sensitivity to pH was tested by using 3-ml aliquots of the samples, adjusted to pH 3.0 with 1.0 N HCl or to pH 13.0 with 1.0 N NaOH, and incubating at room temperature for 30 min. Samples were then neutralized to pH 7.0. A control sample was adjusted for the slight change in volume by adding an equivalent amount of 1.0 M NaCl.

Dialysis of the ascitic fluids was carried out in dialysis tubing (Union Carbide Corp., Chicago, Ill.) for 24 hr against 3 liters of PBS (Grand Island Biological Co., Grand Island, N. Y.).

Approximate molecular size was determined by Diaflo ultrafiltration with Amicon XM 100A, PM 50, and UM 10 mem-
Fluids were considered to have significant lymphocyte-inhibitory blastogenesis by dispensing 40 μl of the whole fluid into triplicate wells of PHA cultures and MLC's at the initial time of stimulation. The cultures were incubated at 37° in an humidified 5% CO2:95% air incubator. On Day 3 of the PHA cultures, Day 4 of the Con A cultures, and Day 6 of the MLC's, 1 μCi of [3H]thymidine (Schwarz/Mann, Orangeburg, N.Y.; specific activity, 1.9 Ci/mmol) was added to each well prior to harvesting on glass fiber filter paper (Grade 934 AH; Reeve Angel, Clifton, N.J.) with a multiple automated sample harvester. The filter paper was dried and processed for liquid scintillation counting.

**General Tissue Culture Methods.** Human peripheral blood lymphocytes were obtained by the following method: 60 ml of defibrinated blood were centrifuged at 1000 × g for 30 min. The buffy coat was removed, diluted 1:1 with RPMI 1640 (Grand Island Biological), layered over an equal volume of lymphocyte separation medium (Litton Bionetics, Kensington, Md.), and centrifuged at 1000 × g for 30 min. The interface, which contained 90 to 95% viable mononuclear cells, was removed and washed 3 times in RPMI 1640. The cells were resuspended at 2.0 × 10^6 cells/ml in RPMI 1640 containing 15% pooled NHS or autologous serum and 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 × 10^5 lymphocytes) and 0.1 ml of MC to each well. Doses of PHA (reagent grade; Burroughs Wellcome and Co., Research Triangle Park, N. C.) or Con A (Pharmacia, Uppsala, Sweden), which had been previously determined to be optimal in our laboratory, were used for mitogen stimulation (0.05 ml/well). In the study of PHA titration, purified PHA (Burroughs Wellcome) was used at the indicated doses as described below.

**Bidirectional MLC's.** MLC's were established by adding 1 × 10^5 lymphocytes from 2 unrelated individuals to wells of the microtiter plate. All tests were done in triplicate, and the cultures were incubated at 37° in an humidified 5% CO2:95% air incubator. On Day 3 of the PHA cultures, Day 4 of the Con A cultures, and Day 6 of the MLC's, 1 μCi of [3H]thymidine (Schwarz/Mann, Orangeburg, N.Y.; specific activity, 1.9 Ci/mmol) was added to each well prior to harvesting on glass fiber filter paper (Grade 934 AH; Reeve Angel, Clifton, N.J.) with a multiple automated sample harvester. The filter paper was dried and processed for liquid scintillation counting.

**Testing of Cystic and Ascitic Fluids.** Cystic and ascitic fluids were screened for their ability to inhibit lymphocyte blastogenesis by dispensing 40 μl of the whole fluid into triplicate wells of PHA cultures and MLC's at the initial time of setup. Included as controls were additional triplicate tests with 40 μl of NHS, 40 μl of PBS, and/or 40 μl of MC. The final volume of all cultures was 0.29 ml. Variation between mean cpm of [3H]thymidine incorporation for NHS, PBS, and MC controls averaged less than 10%. 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:

\[
1 - \frac{\text{Mean cpm of response in test fluid}}{\text{Av. mean cpm of NHS, PBS, and MC controls}} \times 100
\]

Fluids were considered to have significant lymphocyte-inhibitory activity when a greater than 30% inhibition of the lymphocyte blastogenic response with a statistically significant difference (p < 0.01) between the mean cpm of [3H]thymidine uptake for experimental and NHS, PBS, and MC controls was obtained. The 30% level of inhibition represents at least a 3-fold difference compared to the mean variation between control cultures.

**Titration of lymphocyte-inhibitory activity.** Titration of lymphocyte-inhibitory activity in the standard PHA, Con A, and MLC assays was performed by using an arithmetic dilution pattern with the addition of highly active whole AF's (40, 30, 20, and 10 μl/well) at the initiation of the culture (unless indicated otherwise), with the volumes being equalized by addition of PBS (final culture volume, 0.29 ml). In other experiments, titration of activity was carried out similarly except that serum and cell concentrations were varied as specified below. Controls used for these assays were similar to the ones in the screening assays except that a previously determined negative ascitic fluid was often included. In many of the experiments, cells were enumerated and checked for viability by trypan blue exclusion and phase contrast microscopy at the end of the culture period to rule out cytotoxic activity of the inhibitory fluids. Lymphocytotoxicity of the fluids was also assessed in a 51Cr release assay. One × 10^4 PHA-stimulated lymphocytes labeled with 51Cr as previously described (29) were incubated with 30 μl of AF, NHS, and/or MC in triplicate wells of a microtiter plate containing 0.2 ml of MC. After 3 hr, the plates were centrifuged at 500 × g for 5 min, and the supernatant was counted for released 51Cr. Maximum release was obtained by adding 0.1 ml of 1.0 N HCl to control wells containing an identical amount of labeled cells.

**Effect of Inhibitory Ascitic Fluids on Established Cell Lines.** Several inhibitory AF's were tested for their effect on the growth of established cell lines maintained in the Division of Immunology, Duke University, by using a [3H]thymidine incorporation assay. Cell lines that were tested included HSB, a T-lymphoblastoid cell line; SB, a B-lymphoblastoid cell line; HEp-2, a human epithelioma; and an oligodendroglioma cell line. The HSB and SB cell lines grow as a single cell suspension and were harvested by centrifugation. The HEp-2 and oligodendroglioma cell lines were processed by trypsinization (0.25%) (17). The cells were washed 3 times and resuspended in MC as described above. Cells were plated at a concentration of 1 × 10^6 cells/well with 40 μl of inhibitory AF's or NHS added to the cultures. The cultures were incubated for 2 days, after which 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 checked for viability by trypan blue dye exclusion and phase contrast microscopy at the end of the incubation period.

**Statistical Analysis.** Data were analyzed for statistical significance by using the Student t test.

**RESULTS**

**Lymphocyte-inhibitory Activity in Cystic and Ascitic Fluids**

Several whole cystic and ascitic fluids from patients with a variety of ovarian neoplastic diseases were screened for their ability to inhibit PHA and/or MLC responses. The fluids were categorized according to the level of inhibition of the lymphocyte response when tested at the screening dose of 40 μl of fluid per well. The 3 categories were arbitrarily set: negative, 0 to 30% inhibition; intermediate, 30 to 70% inhibition; and high, >70% inhibition. The results of the screening for inhibitory activity along with fluid origin and differential diagnosis are summarized in Table 1. A low frequency (2 of 11) of the cystic...
effusions from primary ovarian tumors was found to have significant lymphocyte-inhibitory activity. One positive cystic fluid was from a patient with Stage I mucinous adenocarcinoma and was found to give 95% inhibition of both the PHA and MLC response of normal lymphocytes. The other positive cystic fluid was from a patient with a benign lesion, mucinous cystadenoma, and was found to have intermediate levels of inhibitory activity in both in vitro assays. In contrast, AF's capable of inhibiting the lymphocyte blastogenic response were found with a much greater frequency (12 of 26). Six of the 12 positive AF's had high levels of inhibitory activity giving >95% inhibition of the PHA and MLC response of normal lymphocytes when tested at the screening dose of 40 μl of ascitic fluid per well. Included in this group of highly active fluids was the pelvic fluid (arbitrarily classified as an AF) from a patient with a benign follicular cyst. Three of the other 6 positive AF's were found to give intermediate levels of inhibition in both in vitro assays (percentage of inhibition of the PHA response, 47.0, 55.0, and 52.5%, respectively; percentage of inhibition of the MLC response, 99.0, 72.3, and 62.9%, respectively). The other 3 positive ascites were found to inhibit only the PHA response of normal lymphocytes (63.9, 30.6, and 64.2% inhibition). Two of these AF's were from patients with endometrial carcinoma, and the other was from a patient with an ovarian serous adenocarcinoma. These 3 fluids, upon repeated testing, were found not to inhibit the MLC response of normal lymphocytes.

The remaining cystic and ascitic fluids from both benign and malignant lesions did not alter the in vitro lymphocyte responses compared to control responses with 40 μl of NHS, PBS, and/or MC added to the culture. At times, minimal inhibition of the PHA response (0 to 30% and not significant with p > 0.05) did occur but upon repeated testing was shown not to be reproducible. In all studies described below, only the fluids showing high levels of inhibition in both PHA and MLC assays were used.

Inhibitory cystic and ascitic fluids were checked for lymphocyte cytotoxicity. A representative experiment with the 51Cr release assay is summarized below. The cpm of released 51Cr for 1 × 10^7 51Cr-labeled, PHA blast target cells incubated with either MC, NHS, and positive AF 526 and/or AF 556 were: 157 ± 11 (S.D.); 148 ± 14; 153 ± 13; and 143 ± 12, respectively. Maximum release was 865 ± 52, obtained by adding 0.1 ml of 1.0 N HCl. By all criteria (see "Materials and Methods"), we could not detect cytotoxic activity in any of the inhibitory fluids.

In Vitro Characterization of Lymphocyte-inhibitory Activity

Dose Dependency. AF's were chosen instead of cystic fluids to characterize the inhibitory activity, primarily because of the frequency of positive fluids, larger volumes, and ease of handling. The dose dependency of the inhibitory activity on the PHA, Con A, and MLC responses of normal lymphocytes is summarized in Table 2, which describes the results from several experiments with AF's having high levels of immunosuppressive activity. A few negative AF's were included for the sake of comparison. The blastogenic response of normal lymphocytes to PHA, Con A, and allogeneic lymphocytes was found to be significantly (p < 0.01) inhibited by the 40, 30, and 20 μl doses of AF's. The data from these experiments also demonstrate that the titration of the inhibitory activity in AF's is in an arithmetic fashion. Generally, inhibition of the blastogenic response approached maximum levels at 20 μl of ascitic fluid per well, and often minimal or no inhibition was observed when the AF's were tested at 10 μl/well (especially in the mitogenic responses). The MLC response in most instances was more sensitive to the effects of the inhibitory activity, as shown by higher levels of inhibition at the lowest dose of AF's tested, than it was in the mitogenic assays.

Preliminary experiments (data not shown) showed that increasing the level of PHA in culture did not overcome the inhibitory effects of the AF's. Maximal inhibition (>95%) of the response over the entire dose range of PHA (0.5, 1.0, 2.0, and 3.0 μg of purified PHA) was observed when lymphocytes were cultured in the presence of 30 μl of the inhibitory AF's.

Effect of Cell Concentration on the Titration of Inhibitory Activity. Increasing the number of cells in culture inversely correlated with the amount of inhibition observed, as shown in Chart 1. This effect was most easily demonstrated at intermediate levels of AF in the culture system. Maximal inhibition of the PHA response was observed at all lymphocyte concentrations when tested with 40 μl of ascites added per culture. At 30 μl of inhibitory fluid, there was a slight decrease in the amount of inhibition only in the cultures with 4 × 10^5 lymphocytes/well. However, at a dose of 20 μl of inhibitory AF, the inhibition of the lymphocyte response to PHA decreased as the cell concentration was increased. The levels of inhibition decreased from 77% at 1 × 10^5 cells/well to 16% at 4 × 10^5 cells/well. The same basic trend was shown for other inhibitory AF's.

### Table 1

<table>
<thead>
<tr>
<th>Origin of fluid and differential diagnosis</th>
<th>No. of fluids observed giving specified levels of lymphocyte inhibition</th>
<th>Frequency of positive fluids</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cystic fluids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous adenocarcinoma</td>
<td>5</td>
<td>0/5</td>
</tr>
<tr>
<td>Mucinous adenocarcinoma</td>
<td>1</td>
<td>1/2</td>
</tr>
<tr>
<td>Endometrial carcinoma</td>
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<td>0/0</td>
</tr>
<tr>
<td>Cystic fibrosarcoma</td>
<td>1</td>
<td>0/1</td>
</tr>
<tr>
<td>Mucinous cystadenoma</td>
<td>1</td>
<td>1/2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>9</td>
<td>1/11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AF's</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian adenocarcinoma</td>
<td>5</td>
<td>1/2</td>
</tr>
<tr>
<td>Papillary adenocarcinoma</td>
<td>2</td>
<td>1/2</td>
</tr>
<tr>
<td>Serous adenocarcinoma</td>
<td>3</td>
<td>1/2</td>
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<tr>
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<tr>
<td>Endometrial carcinoma</td>
<td>1</td>
<td>0/0</td>
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<tr>
<td>Pelvic carcinomatosis</td>
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<td>0/0</td>
</tr>
<tr>
<td>Proliferative endometrium</td>
<td>1</td>
<td>0/0</td>
</tr>
<tr>
<td>Ovarian cyst-pelvic fluid</td>
<td>0</td>
<td>1/1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>14</td>
<td>6/6</td>
</tr>
</tbody>
</table>

a Tested at 40 μl/well.

b Only inhibited the PHA response.

c Two-thirds only inhibited the PHA response.
In vitro titration of the immunosuppressive activity in AF’s from patients with ovarian neoplasms

<table>
<thead>
<tr>
<th>AF</th>
<th>Control response on day tested</th>
<th>Unstimulated lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 μl</td>
<td>30 μl</td>
<td>20 μl</td>
</tr>
<tr>
<td>PHA response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>489</td>
<td>34 ± 37 (99)</td>
<td>148 ± 44 (99)</td>
</tr>
<tr>
<td>501</td>
<td>743 ± 11 (99)</td>
<td>2,418 ± 2,443 (95)</td>
</tr>
<tr>
<td>503</td>
<td>362 ± 169 (99)</td>
<td>130 ± 133 (99)</td>
</tr>
<tr>
<td>507</td>
<td>224 ± 49 (99)</td>
<td>1,191 ± 1,596 (99)</td>
</tr>
<tr>
<td>526</td>
<td>245 ± 192 (99)</td>
<td>217 ± 41 (99)</td>
</tr>
<tr>
<td>495</td>
<td>140,044 ± 2,614 (~)</td>
<td>NT</td>
</tr>
<tr>
<td>485</td>
<td>145,836 ± 4,356 (~)</td>
<td>NT</td>
</tr>
<tr>
<td>Con A response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>489</td>
<td>212 ± 69 (99)</td>
<td>595 ± 277 (99)</td>
</tr>
<tr>
<td>501</td>
<td>303 ± 365 (99)</td>
<td>517 ± 181 (99)</td>
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<td>503</td>
<td>751 ± 579 (99)</td>
<td>563 ± 167 (99)</td>
</tr>
<tr>
<td>495</td>
<td>117,048 ± 3,530 (10)</td>
<td>NT</td>
</tr>
<tr>
<td>485</td>
<td>114,817 ± 6,125 (12)</td>
<td>NT</td>
</tr>
<tr>
<td>MLC response</td>
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<td></td>
</tr>
<tr>
<td>501</td>
<td>466 ± 101 (98)</td>
<td>424 ± 166 (97)</td>
</tr>
<tr>
<td>503</td>
<td>210 ± 193 (97)</td>
<td>210 ± 48 (98)</td>
</tr>
<tr>
<td>507</td>
<td>169 ± 66 (99)</td>
<td>103 ± 71 (99)</td>
</tr>
<tr>
<td>526</td>
<td>30 ± 14 (99)</td>
<td>19 ± 8 (99)</td>
</tr>
<tr>
<td>495</td>
<td>39,137 ± 1,990 (~)</td>
<td>NT</td>
</tr>
<tr>
<td>485</td>
<td>39,366 ± 4,065 (~)</td>
<td>NT</td>
</tr>
</tbody>
</table>

*Mean ± S.D.

**Number in parentheses, percentage of inhibition based on control responses. Dash in parentheses, no inhibition.

†Mean ± S.D. of assays with 40 μl of NHS, 40 μl of PBS, and/or 40 μl of MC added to the culture.

\( ^{a} \) AF's considered not to have lymphocyte-inhibitory activity.

\( ^{b} \) NT, not tested.

**Table 2**

| Serum Concentration and Lymphocyte-inhibitory Activity. Inhibitory AF's were titrated against a standard concentration of lymphocytes (2 × 10^5 cells) in a PHA assay at 4 different levels of NHS concentration—5, 10, 20, and 30%. A representative experiment is illustrated in Chart 2. Maximal inhibition of the lymphocyte response was observed at all concentrations of serum tested when 40 μl of the inhibitory fluid was added to the culture system. At doses of 30 μl, the level of inhibition was slightly decreased in the cultures with 30% serum. However, in cultures with 20 μl of the inhibitory fluid, inhibition of the lymphocyte response inversely correlated with the serum concentration. The levels of inhibition decreased from 78% in cultures with 5% serum to 3% in cultures with 30% serum.

**Temporal Studies.** Addition of 30 μl of an inhibitory AF at the initiation of PHA cultures and MLC's resulted in maximal inhibition of the lymphocyte blastogenic response, as shown in Chart 3. Similar levels of inhibition could be observed when the AF was added at 1.5 hr after initiation of cultures. Addition of the inhibitory AF at 20 and 24 hr after the establishment of the cultures resulted in reduced levels of inhibition of both PHA and MLC responses. Active AF's added 40 and 72 hr after the initiation of PHA and MLC cultures, respectively, resulted in minimal or no inhibitory effects on the lymphocyte blastogenic response.

**Effect of Preincubation with Inhibitory AF's on the Lymphocyte Response.** The next series of experiments were performed to determine the effect of preincubation of lymphocytes with inhibitory AF's on their response to PHA and to characterize the time and temperature dependency of this effect. Two 2 × 10^5 lymphocytes were preincubated with 30 μl of inhibitory AF's in 0.20 ml of MC at 37° for 3, 20, or 24 hr. After the specified time interval, the plates were centrifuged, the supernatant was carefully removed, and the cells were washed once in RPMI 1640 and resuspended in MC and PHA. A representative experiment is illustrated in Chart 4. The lymphocytes that were preincubated for 3 hr in the ascitic fluids with MC showed no alteration in their response to PHA after removal of the inhibitory fluid compared to lymphocytes preincubated in NHS or PBS. However, preincubation of the lymphocytes for 20 and 24 hr in the AF:MC solution resulted in a significant (p < 0.001) decrease in their ability to respond to PHA after removal of the ascitic fluid compared to that of cells preincubated in NHS or PBS and treated similarly.

Chart 5 is representative of experiments in which 2 × 10^6 normal lymphocytes were preincubated for 24 hr with inhibitory AF's at 4 and 37° and then subjected to an incomplete wash (resuspension of the cells in MC and PHA after removal of the AF:MC supernatant) or washed 3 times in PBS prior to resuspension in MC and PHA. The effect of the inhibitory AF's was temperature dependent. Lymphocytes preincubated with the
PHA responsiveness by further incubation in the absence of inhibitory fluids prior to stimulation was assessed. Two \(10^6\) lymphocytes were preincubated with 30-μl amounts of AF’s, NHS, and/or PBS in MC for 24 hr. After this time interval, the plates were centrifuged (1000 \(\times\) g, 5 min); the AF:MC supernatant was carefully removed; and the cells were washed once in RPMI 1640, resuspended in 0.2 ml of MC per well, and incubated for 24 hr at 37° prior to addition of PHA. The results are given in Table 3. After the incubation period in the absence of inhibitory fluid prior to stimulation, the ascitic fluid-preincubated lymphocytes did not regain their ability to respond to PHA. Lymphocytes preincubated with NHS or PBS in MC and

AF at 4° retained their capacity to respond to PHA. In contrast, lymphocytes preincubated with the inhibitory fluids at 37° showed a significant (AF 526, \(p < 0.01\); AF 533, \(p < 0.001\)) reduction in their ability to respond to PHA compared to NHS−, PBS−, and MC-preincubated lymphocytes. This reduced capacity to respond to PHA was not reversed by washing the cells 3 times with PBS. In parallel cultures of the above experiments, a decrease in the number of cells or a loss of viability could not be demonstrated between cells preincubated in AF’s or in NHS, PBS, and MC.

The ability of the ascitic fluid-preincubated cells to recover

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CHART 1. Titration of an inhibitory AF against 4 different lymphocyte concentrations in response to PHA. The data are presented as the percentage of inhibition based on control responses. The control responses for 1, 2, 3, and 4 \(\times 10^5\) lymphocytes/well in cpm of \(^{3}\)H)thymidine were: 38,482 ± 4,380 (S.D.); 106,449 ± 4,484; 160,069 ± 6,137; and 183,698 ± 5,364, respectively. Values of \(^{3}\)H)thymidine uptake for unstimulated lymphocytes never reached above 500 cpm for each concentration of lymphocytes used.

CHART 2. Effect of increasing the concentration of NHS on the titration of the lymphocyte-inhibitory activity in AF’s. The data are presented as the percentage of inhibition based on control responses. Mean cpm of \(^{3}\)H)thymidine uptake for control response of 2 \(\times 10^5\) lymphocytes/well in 5, 10, 20, and 30% NHS were: 103,329 ± 2,042 (S.D.); 126,893 ± 3,690; 130,156 ± 22,692; and 128,133 ± 6,742, respectively. Unstimulated lymphocytes averaged 309 ±75 cpm of \(^{3}\)H)thymidine uptake.

CHART 3. Effect of adding 30 μl of an inhibitory AF per well at various intervals of ongoing PHA cultures and MLC’s on the lymphocyte response. The data are presented as the percentage of inhibition based on control responses. Mean cpm of \(^{3}\)H)thymidine uptake for the control PHA and MLC responses were: 175,777 ± 4,814 (S.D.) and 53,748 ± 8,099, respectively. Unstimulated lymphocytes averaged less than 1,300 cpm of \(^{3}\)H)thymidine uptake in both experiments.

CHART 4. Time dependency of the inhibitory AF’s to effect inhibition of the lymphocyte response. Two \(\times 10^5\) lymphocytes/well were preincubated with 30 μl of inhibitory AF’s, PBS, or NHS with MC in microtiter plates. After the specified time interval, the plates were centrifuged at 1000 \(\times\) g for 5 min; the supernatant was carefully removed, and the cells were washed once with 0.25 ml RPMI 1640, resuspended in MC (0.2 ml), and stimulated with PHA. The data are expressed as the mean cpm of \(^{3}\)H)thymidine uptake; bars, S.D.
treated similarly responded.

To determine whether the preincubation of lymphocytes with AF's removed the inhibitory activity in these fluids, 5 x 10^6 lymphocytes were incubated with 0.5 ml of inhibitory AF's at 4 and 37°C. The number of lymphocytes required to absorb a significant portion of inhibitory activity from 0.5 ml AF’s was estimated by direct inference from experimental data, as described in Chart 1. It was estimated that approximately (3 to 4 x 10^6 lymphocytes/20 µl) 7.5 to 10 x 10^6 lymphocytes were required to completely absorb the inhibitory activity in 0.5 ml of AF. In the presently described absorption experiments, 0.5 ml of AF's was slightly greater than a limiting dose. After 24 hr of incubation, the suspension of cells and AF was centrifuged at 1000 x g for 30 min. The supernatant was then tested for its ability to inhibit freshly isolated lymphocytes in a PHA assay; results are shown in Chart 6. Incubation of cells with ascitic fluid at 4°C resulted in only a marginal loss of inhibitory activity. In contrast, the AF incubated with the lymphocytes at 37°C absorbed a significant (40 µl, p < 0.05; 30 µl, p < 0.001; 20 µl, p < 0.01; 10 µl, p < 0.01) amount of inhibitory activity compared to the control fluid incubated at 37°C. Absorption of the lymphocyte-inhibitory activity from the AF's did not result in a significant alteration of the protein concentration compared to that of control samples which did not absorb the activity. The data would indicate that the active moiety was a minor component if the lymphocyte-inhibitory factor was assumed to be a protein.

Effect of Inhibitory AF’s on Established Cell Lines. Highly active lymphocyte-inhibitory fluids were tested for their effect on [3H]thymidine incorporation by established cell lines. The results are summarized in Table 4. A highly significant (p < 0.001) decrease in cpm of [3H]thymidine incorporation was observed when 1 x 10^6 HSB or SB cells, derived from T- and B-lymphoblastoid cell lines, respectively, were cultured with 40 µl of the active AF's compared to control cultures with 40 µl of NHS added. In contrast, no significant inhibition of [3H]thymidine incorporation was observed when 1 x 10^6 Hep2 and oligodendroglioma cells were cultured with the AF’s. Viability testing by trypan blue dye exclusion and phase contrast mi-
estimation by Diaflo ultrafiltration revealed that the majority of cell lines. Preclinical studies did not reveal a cytotoxic effect of the AF's on the cell lines.

**Preliminary Biochemical Characterization of the Lymphocyte-inhibitory Activity in AF's.** Preliminary biochemical characteristics of the lymphocyte-inhibitory activity found in the AF's were determined as given in Table 5. Molecular size estimation by Diaflo ultrafiltration revealed that the majority of the inhibitory activity resided in the fraction which passed through the XM 100A and was retained by the PM 50 filters. This fraction significantly (p < 0.001) inhibited the PHA response of normal lymphocytes compared to the control responses. There was some slight but significant (p < 0.01) inhibition of the PHA response by the fraction retained by the XM 100A filter, which may be due to incomplete ultrafiltration.

Lymphocyte-inhibitory activity was tested for its ability to withstand pH extremes. Adjustment of the fluids to pH 3.0, followed by neutralization, resulted in a slight loss of activity as shown by a significant (p < 0.001) increase in the lymphocyte response to PHA in the presence of the treated fluid compared to the response in the volume-adjusted control. Alkaline treatment (pH 13.0) resulted in a 43% reduction in the ability of the ascitic fluid to inhibit the PHA response of normal lymphocytes.

Heat stability was assessed, and the results are shown in Table 5, Experiment B. The inhibitory activity withstood heating to 56°C for 30 min as indicated by no significant difference in the inhibition of the PHA response compared to that of the untreated original fluid. Heat treatments of 62 and 100°C resulted in significant reductions (p < 0.05 and < 0.001, respectively) in the ability of the AF’s to inhibit the PHA response, with the 100°C-treated fluid losing the majority of its activity.

Active AF’s were dialyzed against 3 liters of PBS for 24 hr and then tested for ability to inhibit the PHA response of lymphocytes. The results are given in Table 5, Experiment B. Dialysis of the active fluids did not result in a significant loss of inhibitory activity compared to that of the original, untreated fluid.

**Discussion**

The present studies, in which cystic and ascitic effusions from patients with benign and malignant ovarian neoplastic disease were tested for ability to inhibit in vitro lymphocyte responses, have confirmed and extended the studies of Badger et al. (3, 4), which demonstrated that ascitic fluids from cancer patients with peritoneal metastases contain a soluble factor(s) which nonspecifically inhibits in vitro lymphocyte function. A substantial percentage of AF’s from the patients in the present study were found to contain a noncytotoxic, nondialyzable humoral factor(s) which inhibited lymphocyte blastogenesis. Nine of the 12 positive AF’s inhibited both the PHA and MLC responses of normal lymphocytes, with the MLC response being, in general, more sensitive to the effects of the inhibitory activity contained in these fluids. The enhanced sensitivity of the MLC response to serum- and ascites-inhibitory factors has been documented in other systems (3, 6, 22). The 3 remaining positive AF’s were found to inhibit only the lymphocyte response to PHA. These findings could be explained by a factor capable of inhibiting only a subpopulation of lymphocytes responsive to PHA since recent studies have shown that MLC-
responsive cells are not included in the subpopulation of lymphocytes which respond to PHA (13). However, competitive binding of the lectin with the glycoproteins as suggested by Yachnin (35) and by Miller (22) cannot be ruled out.

The present studies have also demonstrated that some cystic effusions of primary ovarian tumors will inhibit PHA and MLC responses of normal lymphocytes. These inhibitory cystic fluids were found to occur in a much lower frequency (2 of 11) when tested at the screening dose of 40 µl of whole fluid. The negative cystic fluids and AF's may have contained the inhibitory factor(s) but in levels too low to detect in the present systems, especially when the exquisite concentration dependence of the inhibitory activity is considered. The finding of lymphocyte-inhibitory activity in the effusions of primary ovarian tumors lends some support to the hypothesis that tumor cells produce or induce the formation of factors which are capable of modulating lymphocyte function effecting local immunosuppression (3, 8).

In these 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 (mucinous cystadenoma; pelvic fluid-follicular cyst). In contrast to our findings, Badger et al. (3) were not able to detect lymphocyte-inhibitory activity in AF's from several patients with nonneoplastic disorders. This discrepancy could be explained by differences between the pathophysiological origins of the control ascitic fluids tested originally by Badger et al. (3) (i.e., congestive heart failure; sterile pleural effusions) and the effusions associated with benign ovarian disorders tested in the present studies. Although a recent report by Badger et al. (2) indicates that lymphocyte-inhibitory activity was found in ascitic effusions from patients with diseases not related to cancer, in support are findings of LeBien et al. (16), which suggest that the presence of lymphocyte-antiproliferative factors in murine AF's may constitute a natural host-mediated response to inflammatory stimuli which is not limited to malignant neoplasia. One cannot rule out the possibility that both benign and malignant tumor cells may produce factors which modulate immune responsiveness. Recent evidence has demonstrated that α-fetoprotein is immunosuppressive in vivo and in vitro. Preliminary results (data not shown) would indicate that α-fetoprotein was not found in the cystic and ascitic effusions tested in the present studies as determined by double immunodiffusion in agar techniques. In the studies described by Badger et al. (3), the concentration of α-fetoprotein in the fluids containing lymphocyte-inhibitory activity was less than 10 ng/ml, far below the effective inhibitory levels demonstrated for α-fetoprotein (23). The origin and nature of the lymphocyte-inhibitory factor(s) found in the effusions associated with benign and malignant ovarian neoplasms and their relation to the immunosuppressive factors described in serum (1, 5, 12, 22, 26) must await complete isolation and biochemical characterization.

The factor(s) responsible for effecting lymphocyte inhibition demonstrated an exquisite concentration dependence approaching an almost all or none phenomenon. Maximal inhibition of the lymphocyte response occurred when the active AF's were added within 24 or 40 hr of initiation of PHA and MLC cultures, respectively, suggesting that the inhibitory component works on the early stages of lymphocyte activation. The inhibitory effects of the AF's were found to be dependent on the lymphocyte and serum concentrations used in the assay system and could account in part for the arithmetic titration pattern and concentration dependency of the inhibitory factor(s). These data would also suggest that the sensitivity of the assay systems in detecting lymphocyte-inhibitory activity could be increased by decreasing the concentrations of serum and lymphocytes and/or increasing the dose of AF's in the initial culture system. Increased sensitivity of the proliferation assays would allow for the detection of AF's with low levels of inhibitory activity not detectable in the standard assay system described.

The mitigation of lymphocyte inhibition by NHS suggests that there is a factor capable of neutralizing the effects of the inhibitory factor found in AF's, although we cannot exclude the possibility that the serum effect described in the present studies represents a nonspecific adsorption to serum proteins. However, in support of these observations are the results of Ting (30) and Ting et al. (31), which show the presence of a serum component that abrogates the effect of immunosuppressive activity found in murine AF's. They postulated that these "counterfactors" may play a role in the control of tumor growth. The presence of putative counterfactors in serum may account for the inability to demonstrate any measurable lymphocyte-inhibitory activity in the sera of cancer patients with highly inhibitory AF's as reported by Badger et al. (3) and confirmed in our studies (data not shown). It is not known, however, if the sera from these cancer patients have the capacity to neutralize the factor(s) responsible for the inhibition of lymphocyte function. It is possible that the general impairment of immune responsiveness often observed in patients with advanced neoplastic disease (10, 11, 21) may represent a failure of putative control mechanisms to regulate lymphocyte-antiproliferative factors. The chronic or excessive production of lymphocyte-inhibitory factor(s) in cancer patients as postulated by Nimberg et al. (25) may in effect overwhelm the counterfactors contained in serum and lead to a failure of control mechanisms, accounting for a state of impaired immune reactivity. The role of serum counter factors to lymphocyte-inhibitory activities in cancer patients bears the need for further assessment and is currently under investigation.

Our studies have also demonstrated that the inhibitory activity is absorbed and/or metabolized by the lymphocyte:monocyte cell suspension to effect inhibition of the blastogenic response. This process was found to be both time and temperature dependent, suggesting that metabolically active cells were required to absorb the inhibitory component from the AF's.

The molecular size of the nondialyzable inhibitory factor was estimated by Diaflo ultrafiltration to be between 50,000 and 100,000 daltons. The size estimation would be accurate if the inhibitory factor was assumed to be a globular protein. At present, the nature of the active component is not known. However, the inhibitory activity in AF's from ovarian cancer patients was similar in size to the active component described in murine AF's and in pleural effusions of breast cancer patients estimated by gel filtration and SDS-polyacrylamide gel electrophoresis (31). The possibility remains that the active factor is a small molecule which is tightly bound to a protein of the apparent molecular size as found for the peptide that is associated with immunoregulatory α-globulin (26).

In the present studies, lymphocytes preincubated in the active fluids did not regain their ability to respond even after a
mild washing with and with a 24-hr incubation prior to stimulation. More extensive washing may be required to allow the cells to recover, as suggested by the studies of Mannick et al. (18) in which the PHA response of lymphocytes from cancer patients could be enhanced when the cells were washed 6 times. The inability to recover from the effects of the inhibitory activity in AF’s may account for the depressed function of peripheral blood lymphocytes often observed in cancer patients (18). Recirculating lymphocytes may lose their capacity to respond when they migrate through effusions which contain high levels of the inhibitory factor(s). To verify this hypothesis, the functional capacity of the lymphocytes in the fluid and/or tissues which are found to contain the inhibitory activity, as well as the immune responsiveness of peripheral blood lymphocytes in patients with inhibitory effusions, must be assessed.

In summary, a substantial percentage of AF’s and some cystic effusions from patients with benign and malignant ovarian neoplastic diseases were found to contain a nondialyzable, noncytotoxic factor(s) which inhibited PHA and MLC responses of normal lymphocytes. The preliminary findings that active fluids inhibited the proliferation of HSB (T-) and SB (B-) lymphoblastoid cell lines as measured by [3H]thymidine incorporation but did not have any effect on the HEP-2 and oligodendroglioma cell lines suggest that there was a selective effect on cells of lymphoid origin. However, other established cell lines are being tested to verify the selective nature of lymphoid cell inhibition by the AF’s. Investigations are also currently underway to purify and characterize the active factor(s) with respect to its physical and chemical properties and to determine if the inhibitory activities associated with the AF’s are attributable to a single factor.

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Inhibition of \emph{in Vitro} Lymphocyte Function by Cystic and Ascitic Fluids from Ovarian Cancer Patients

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