Partial Purification and Characterization of a Lymphocyte-inhibitory Factor(s) in Ascitic Fluids from Ovarian Cancer Patients

Allan D. Hess, Stanley A. Gall, and Jeffrey R. Dawson

Division of Immunology, Department of Microbiology and Immunology [A. D. H., J. R. D.], and Department of Obstetrics and Gynecology [S. A. G.], Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT

Attempts were undertaken to purify and characterize the lymphocyte-inhibitory activity found in the ascitic effusions from ovarian cancer patients. The active moiety responsible for the inhibition of in vitro lymphocyte function was partially purified from several ascitic fluids by means of concanavalin A-Sepharose 4B (negative) affinity and DE52 ion-exchange chromatography, with final yields of 68 to 74% and 3- to 8-fold increases in specific activity. Analytical polyacrylamide gel electrophoresis showed that the active fraction contained mostly albumins and α-globulins. Further purification by semi-preparative electrophoresis in polyacrylamide gels revealed that the majority of the lymphocyte-inhibitory activity migrated with the albumins, although minor levels of activity were detected in the material which eluted from the α-globulin region. The lymphocyte-inhibitory activity was found to be separable from albumin by means of a rabbit anti-human serum albumin immunoadsorbent column. The active factor which lost activity upon storage at −20° was found to have a molecular size of 40,000 to 80,000 daltons, as estimated by Sephadex G-200 filtration. Attempts to separate an active, small-molecular-size component from fractions containing the inhibitory activity by acid ultrafiltration were largely unsuccessful. Immunodiffusion analysis of the purified fractions following removal of albumin revealed the presence of contaminating amounts of α1-acid glycoprotein and α1-antitrypsin. However, levels of these two proteins did not correlate with the presence of lymphocyte-inhibitory activity.

INTRODUCTION

Normal serum from various species has been shown to contain components which nonspecifically modulate immune responses (1, 5, 7–9, 16, 30, 43). Occhino et al. (34) have described an immunoregulatory peptide that is noncovalently linked to an α-globulin and is found in low quantities in normal human serum. Recent findings suggest that this peptide may regulate T-lymphocyte function in vitro and in vivo (9, 10, 34). Similarly, Nelken et al. (18, 32) have isolated 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 (37, 44).

Recent studies have demonstrated that advanced neoplastic disease is often associated with a general impairment of immune function (15, 17, 29). High levels of serum immunoregulatory factors which nonspecifically inhibit in vitro and in vivo lymphocyte function (13, 17, 21, 25, 28, 39, 40, 45) are thought to account for the depressed immune status of anergic cancer patients (13, 33). Furthermore, 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 has been well documented (2–4, 19, 20, 27, 40–42, 46). It has been postulated that tumor cells may locally produce or induce the formation of factors capable of inhibiting lymphocyte function, thus effecting local immunosuppression (3, 4). Hess et al. (19) have demonstrated that a substantial number of ascites fluids from ovarian cancer patients contained a nondialyzable, nontoxic factor(s) with an apparent molecular size of 50,000 to 100,000 daltons which inhibited the in vitro response of normal lymphocytes. However, the presence of lymphocyte-inhibitory activity was not limited to effusions of malignant origin but was also found in a few benign ovarian disorders, suggesting that the presence of lymphocyte antiproliferative factors in these fluids may constitute a natural host-mediated response to inflammatory stimuli as postulated by LeBien et al. (22). The relation of the lymphocyte-inhibitory factors found in ascitic effusions to those described in serum remains unclear. The present investigations were undertaken to purify and characterize the lymphocyte-inhibitory factor(s) found in ascitic effusions from ovarian cancer patients.

MATERIALS AND METHODS

Preparation of Samples. Ascitic fluids were obtained during surgery at Duke University Medical Center from patients suspected of having ovarian cancer. Fluids were clarified by centrifugation (1800 × g for 30 min), aliquotted, and stored at −20° as described previously (19).

Chromatographic Materials and Techniques. Sephadex G-25, Sephadex G-200, Sepharose 4B, and Con A-S-4B3 were obtained from Pharmacia Fine Chemicals, Piscataway, N. J. DE52 was purchased from Whatman Inc., Clifton, N. J., and Affi-Gel Blue was obtained from Bio-Rad Laboratories, Richmond, Calif. The Sephadex G-200 column was calibrated with blue dextran (Pharmacia), the serum proteins IgG and albumin, and 51Cr as sodium chromate (Amersham/Searle Corp., Arlington Heights, Ill.). Ascites fluids clarified by centrifugation (19).

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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.

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or partially purified fractions were chromatographed at 4°, and
the fractions were assayed for \( A_{\text{480 nm}} \). Fractions under
the corresponding protein peaks were pooled, and buffers were
exchanged either by chromatography over Sephadex G-25 or
by ultrafiltration on UM-10 membranes (Amicon Corp., Lexing-
ton, Mass.) using a solution of 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 to approximately 75
to 80% of their original volume, sterile filtered through a 0.22-
\( \mu \)m Millex filter (Millipore Corp., Bedford, Mass.), and tested
for inhibitory activity (19).

**Lipoprotein Isolation.** Lipoproteins were isolated from whole
ascites fluids and Fraction D (Table 1) by density gradient
flostation as previously described (12). The samples were ad-
justed to a density of 1.21 g/ml by addition of solid KBr and
centrifuged at 130,000 x g for 40 hr at 16—18°. The lipopro-
ten-enriched fraction and the nonlipoprotein fraction were
chromatographed over Sephadex G-25, using 0.15 M NaCl:
phosphate (pH 7.0) as the exchange buffer, and concentrated by Amicon ultrafiltration.

**Semipreparative Electrophoretic Purification.** Fraction D
(Table 1) was subjected to semipreparative polyacrylamide gel
electrophoresis using the discontinuous buffer system de-
scribed by Maizel (23). Protein (1 to 2 mg) was mixed with an
equal volume of 10% glycerol and 0.02% phenol red, layered
onto each of thirty-six 0.85- x 7-cm 6.5% polyacrylamide gels
(acrylamide:bisacrylamide, 30:0.8) with a 1-cm 3% polyacryl-
amide gel stacking system. Electrophoresis was carried out at
4° for 6 to 8 hr at 2.5 ma/gel with 0.04 M Tris-glycine as the
electrode buffer (pH 8.3). Protein bands were localized by
staining one gel in Coomassie blue and perchloric acid for 20
min was determined by single radial immunodiffusion (24) in
immunodiffusion plates with normal human serum standards
preweighed amounts of twice crystallized albumin as the stand-
ards. \( \alpha_1 \)-Antitrypsin and \( \alpha_2 \)-acid glycoprotein were determined
by single radial immunodiffusion using commercially prepared
immunodiffusion plates with normal human serum standards
(M-partigen kits; Calbiochem-Behring Corp., La Jolla, Calif.).

**Acid Ultrafiltration.** Electrophoretically purified samples
containing lymphocyte-inhibitory activity were adjusted to pH
3.5 by 5- to 10-fold dilution with 0.01 M acetic acid: 0.15 M

---

**Table 1**

<table>
<thead>
<tr>
<th>Ascertes Fluid</th>
<th>Sample volume</th>
<th>Protein concentration (mg/ml)</th>
<th>Minimum mg protein for &gt;90% inhibition of the PHA responsea</th>
<th>Specific activity (units/mg)</th>
<th>Total activity (units)</th>
<th>Fold purification</th>
<th>% of Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>526. Clarified ascites fluid</td>
<td>40</td>
<td>44.8</td>
<td>0.90</td>
<td>1.11</td>
<td>1989</td>
<td>2.3</td>
<td>90.3</td>
</tr>
<tr>
<td>Con A effluent (Fraction A)</td>
<td>36</td>
<td>19.5</td>
<td>0.39</td>
<td>2.56</td>
<td>1797</td>
<td>4.5</td>
<td>68.6</td>
</tr>
<tr>
<td>Con A effluent-DE52-eluted (Fraction D)</td>
<td>10</td>
<td>27.3</td>
<td>0.20</td>
<td>5.00</td>
<td>1365</td>
<td>7.9</td>
<td>71.8</td>
</tr>
<tr>
<td>503. Clarified ascites fluid</td>
<td>40</td>
<td>44.9</td>
<td>1.35</td>
<td>0.74</td>
<td>1329</td>
<td>2.5</td>
<td>82.7</td>
</tr>
<tr>
<td>Con A effluent (Fraction A)</td>
<td>22</td>
<td>26.6</td>
<td>0.53</td>
<td>1.88</td>
<td>1100</td>
<td>7.9</td>
<td>71.8</td>
</tr>
<tr>
<td>Con A effluent-DE52-eluted (Fraction D)</td>
<td>7</td>
<td>23.2</td>
<td>0.17</td>
<td>5.88</td>
<td>955</td>
<td>3.2</td>
<td>73.8</td>
</tr>
<tr>
<td>533. Clarified ascites fluid</td>
<td>22</td>
<td>32.2</td>
<td>0.64</td>
<td>1.56</td>
<td>1105</td>
<td>3.2</td>
<td>73.8</td>
</tr>
<tr>
<td>Con A effluent (Fraction A)</td>
<td>6.0</td>
<td>27.2</td>
<td>0.20</td>
<td>5.00</td>
<td>816</td>
<td>3.2</td>
<td>73.8</td>
</tr>
<tr>
<td>Con A effluent-DE52-eluted (Fraction D)</td>
<td>NTa</td>
<td>TV</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>V</td>
</tr>
</tbody>
</table>

a Estimated by \( A_{\text{480 nm}} \).

b Arbitrarily defined as one unit of inhibitory activity.

c NT, not tested. Ascites Fluid 533 was fractionated by batch preparation, and Fraction A material was processed immediately and not tested.
Wellcome and Co., Research Triangle Park, N. C.) were used.

Culture Medium 1640, 15% pooled normal human serum 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 (1.5 × 10^6 lymphocytes) and 0.1 ml of complete medium to each well. Two μg of PHA (reagent grade; Burroughs Wellcome Co., Research Triangle Park, N. C.) were used for mitogen stimulation in a volume of 0.05 ml/well (final culture volume, 0.25 ml/well). Bidirectional MLC were established by adding 1.5 × 10^6 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°C in a humidified 5% CO2 incubator. On Day 3 of the PHA 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 16 to 18 hr prior to harvesting onto glass fiber filter paper (Grade 934 AH; H. Reeve Angel & Co., Inc., Clifton, N. J.) with a multiple automated sample harvester. The filter paper was dried and processed for liquid scintillation counting.

Inhibition of the PHA and MLC proliferative responses by clarified ascites fluids or partially purified fractions was determined and quantitated with respect to the PHA and MLC response of control cultures as described previously (19). Ascitic fluids and/or protein fractions were added (40 μl/well) to triplicate wells of both PHA and MLC cultures at the initiation of culture. The control PHA and MLC cultures were additional triplicate tests to which 40 μl of normal human serum, 40 μl of Dulbecco’s PBS, 40 μl of complete medium, and/or 40 μl of buffer were added. Results are expressed as the mean cpm (±S. D.) of [3H]thymidine uptake. The percentage of inhibition was calculated according to the following formula:

\[
\% \text{ of inhibition} = \frac{1 - \text{av. mean cpm of PHA and/or MLC response with added test fluid or protein fraction}}{\text{mean cpm of PHA and/or MLC response with added normal human serum, Dulbecco’s PBS, complete medium, and/or buffer}} \times 100
\]

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

**RESULTS**

**Lymphocyte-inhibitory Ascites Fluids.** Twelve of 26 ascites fluids from patients with benign and malignant ovarian disease were found to contain a noncytotoxic factor(s) which inhibited the in vitro blastogenic response of normal lymphocytes as described previously (19). The inhibitory titer and its loss of activity upon storage at −20°C of a representative inhibitory ascites fluid is shown in Chart 1. Test samples were derived from aliquots of the original fluid clarified by centrifugation and were never thawed and refrozen more than once. Similar titration patterns of inhibition and decay of inhibitory activity upon storage at −20°C were observed when the fluids were tested in the MLC assay.

**Con A-S-4B and DE52 Ion-Exchange Chromatography of the Lymphocyte-inhibitory Activity in Ascites Fluids.** Clarified ascites fluids containing lymphocyte-inhibitory activity were chromatographed on a Con A-S-4B column (developing buffers: 0.03 M NaCl:0.01 M potassium phosphate buffer, pH 7.0; or Dulbecco’s PBS which contained 10^−3 M Mg2+ and Ca2+ chloride salts), dividing the sample into 2 protein fractions (Fractions A and B). The proteins which were bound to the Con A-S-4B column were eluted with 10% dextrose in Dulbecco’s PBS and labeled Fraction B. The Con A-S-4B effluent fraction (Fraction A), which was found to contain the majority of the lymphocyte-inhibitory activity, was then chromatographed on DE52 in 0.03 M NaCl:0.01 M potassium phosphate buffer (pH 7.0). Material which did not bind to the resin (effluent or...
aliquoted, and stored at —20°. After various intervals of storage, as indicated, ascites fluids. A lymphocyte-Inhibitory ascites fluid was clarified by centrifugation, of Inhibition based on control responses. The control PHA responses for Days PHA response of normal lymphocytes. The data are presented as the percentage of (3H\textsubscript{J}thymldine uptake for unstimulated lymphocytes (without PHA) averaged 30, 72, 118, and 133, in cpm of [3H]thymidine, were 86,876 ± 1,148 (S.D.), 102,948 ± 5,236, 94,490 ± 2,929, and 63,364 ± 2,177, respectively. Values of [3H]thymidine uptake for unstimulated lymphocytes (without PHA) averaged less than 500 cpm on each day tested.

Fraction C) showed little inhibitory activity. Material which absorbed to the ion-exchange column under these conditions was desorbed with 1.0 M NaCl and found to contain the lymphocyte-inhibitory activity (Fraction D). In subsequent studies, elution of DE52 columns with 0.35 M NaCl resulted in fractions with levels of inhibitory activity similar to those of Fraction D. However, stepwise elution using narrow molarity ranges of NaCl and/or a continuous linear gradient resulted in substantial losses of inhibitory activity.

Table 1 summarizes the fold purification, specific activities, and percentage of yield of the lymphocyte-inhibitory activity in 3 ascites fluids after chromatographic separation on Con A-S-4B and DE52. The minimum amount of protein added per well resulting in a >90% inhibition of the lymphocyte response to PHA (arbitrarily defined as a unit of lymphocyte-inhibitory activity) for Ascitic Fluid 526 decreased from 0.90 mg/well for the original fluid to 0.39 mg/well for the Con A effluent fraction and 0.20 mg/well for the DE52-eluted material. The relative specific activities (inhibitory units/mg) of these fractions were 1.11, 2.56, and 5.00, respectively, accounting for a 4.5-fold purification. Similar increases in specific activities were observed for the Con A effluent and DE52-eluted fractions of inhibitory Ascites Fluids 503 and 533 (Table 1), with 7.9- and 3.2-fold purifications, respectively. The percentage of yield ranged from 82 to 90% after Con A-S-4B chromatography and 68 to 74% after chromatographic separation on DE52. Similar results were obtained for 2 other ascites fluids from ovarian cancer patients and a pelvic fluid from a patient with a benign follicular cyst.

Two samples of pooled normal human serum and one sample of an ascitic fluid not considered to have lymphocyte-inhibitory activity were fractionated according to a schema identical to that for the inhibitory ascites fluids. Fraction D, as well as Fractions A, B, and C from 2 different samples of normal human serum, did not significantly inhibit the PHA and MLC responses of normal lymphocytes. Fraction D of an ascites fluid that was considered negative was found to inhibit the lymphocyte response slightly in both assays (36 and 29% inhibition of the PHA and MLC responses, respectively).

Molecular Size Estimation of the Lymphocyte-inhibitory Activity by Sephadex G-200 Gel Filtration. Five-m1 samples of Fraction D containing lymphocyte-inhibitory activity were chromatographed on Sephadex G-200 (Chart 2A). Fractions were pooled as illustrated in Chart 2A, concentrated to a final volume of 3 ml each, and tested for ability to inhibit PHA and MLC responses (Chart 2B). G-200 Pools IV and V were found to significantly inhibit (p < 0.01) both PHA and MLC responses. No significant inhibitory activity was observed in G-200 Pools I to III. G-200 Pool VI was found to slightly (p < 0.05) inhibit the MLC response but did not have a significant effect on the PHA response.

Semipreparative Electrophoretic Purification of the Lymphocyte-inhibitory Activity. Fraction D was applied to thirty-six 0.85- x 7-cm 6.5% polyacrylamide gels (1.0 to 2.0 mg/gel).
and electrophoresed. After completion of the run, gels were sectioned and pooled according to the general format as diagrammed in Chart 3A. Proteins eluted from the gel sections were tested for lymphocyte-inhibitory activity in the PHA and MLC assays, and the results are shown in Chart 3B. The majority of the lymphocyte-inhibitory activity was found to be associated with the material which eluted from the albumin (SP II) region as noted by significant inhibition of both PHA (99%, p < 0.001) and MLC (99%, p < 0.001) responses. Material eluted from the SP IV region was found to cause slight but significant inhibition of both PHA (18%, p < 0.01) and MLC (35%, p < 0.01) responses of normal lymphocytes.

Similar results were obtained when Fraction D from other lymphocyte-inhibitory ascites fluids were subjected to semipreparative electrophoresis. The material that eluted from the albumin region (SP II) was consistently found to contain the majority of the lymphocyte-inhibitory activity, and the SP IV region was usually associated with low but significant levels of activity. Fractions SP III and SP V did not consistently show significant levels of lymphocyte-inhibitory activity. SP I was occasionally found to contain minor levels of activity which could have been due to contamination with material from the SP II region.

Fraction D from normal human serum was also subjected to semipreparative electrophoresis and similarly fractionated. Material eluted from the albumin region and from the remainder of the gel was not found to inhibit lymphocyte responses.

**Affi-Gel Blue Chromatography.** Samples of whole ascites fluids and purified preparations (Fraction D) were chromatographed on an Affi-Gel Blue column, which has strong affinity for albumins, lipoproteins, and nucleotide-requiring enzymes (6). Proteins bound to the column were eluted with 1.4 M NaCl: 0.01 M potassium phosphate buffer (pH 7.0). The effluent and eluted fractions from Affi-Gel Blue were tested for their ability to inhibit the lymphocyte response to PHA. Chromatography of lymphocyte-inhibitory fluids and purified fractions on Affi-Gel Blue columns resulted in severe losses of activity (data not shown). The effluent material from both whole fluids and purified preparations was not active. Eluted fractions from whole fluids had minimal lymphocyte-inhibitory activity as noted by slight inhibition of the PHA response (less than 30%). Elution with base (0.05 M Tris:HCl, pH 8.5), 10 mM NAD, or 2.0 M KCl did not result in any substantial recovery of lymphocyte-inhibitory activity.

**Lipoprotein Fractionation.** Lipoprotein-enriched and lipoprotein-poor fractions were obtained from samples of clarified ascites fluids and Fraction D by flotation in a density gradient (density, 1.21 g/ml) and tested for the presence of lymphocyte-inhibitory activity. The results of representative experiments are shown in Table 2. The lipoprotein-enriched fractions from the whole ascites fluid and from purified Fractions D were not found to inhibit the lymphocyte response compared to control cultures. Minimal inhibition of the PHA response was observed when the lipoprotein-enriched fraction from normal human serum was added to the culture. In contrast, addition of lipoprotein-poor fractions from the ascites fluid and/or Fraction D to the cultures resulted in significant inhibition of the PHA response. The nonlipoprotein fraction of normal human serum did not show any lymphocyte-inhibitory activity when tested in a similar fashion.

**Removal of Albumin from Inhibitory Fractions by Immunoabsorption.** SP II samples (1.4 to 2.0 ml) were chromatographed on a rabbit anti-HSA-Sepharose 4B column. The bound albumin was eluted with 0.05 M Tris:0.15 M NaCl (pH 11.5). The effluent and eluted fractions were tested for lymphocyte-inhibitory activity (Table 3). Effluent material was found to significantly (p < 0.001) inhibit the PHA response of normal lymphocytes. Inhibition of the MLC response was also seen when the anti-HSA effluent fractions were added to the culture system. Although loss of activity was apparent, the inhibitory activity per mg increased (SP II-A, 26% inhibition per 0.47 mg; SP II-A anti-HSA effluent, 72% inhibition per 0.16 mg; SP II-B, 36% inhibition per 0.418 mg; and SP II-B anti-HSA effluent, 68% inhibition per 0.176 mg). Slight inhibition of the lymphocyte response was observed when the eluted albumin fraction from SP II-A was added to the culture system. However, it was significantly (p < 0.001) less than the levels found in the effluent fraction.

Although the effluent fraction contained small quantities of
albumin (3.6 and 4.4 mg/ml estimated by radial immunodiffusion), the immunoabsorption column effectively removed >90% of the total albumin from both SP II preparations (61.69 and

Table 2
Lipoprotein fractionation of lymphocyte-inhibitory ascites fluid and partially purified Fraction D

<table>
<thead>
<tr>
<th>Protein concentration of fraction (mg/ml)</th>
<th>PHA response (cpm [³H] thymidine uptake)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>526. Fraction D (Con A effluent DE52-eluted)</td>
<td>20.6</td>
<td>467 ± 574 (99)</td>
</tr>
<tr>
<td>Lipoprotein-enriched fraction</td>
<td>2.0</td>
<td>80,132 ± 4,645</td>
</tr>
<tr>
<td>Nonlipoprotein fraction</td>
<td>18.1</td>
<td>256 ± 159 (99)</td>
</tr>
<tr>
<td>544. Clarified ascites fluid</td>
<td>49.6</td>
<td>11,268 ± 1,748 (85)</td>
</tr>
<tr>
<td>Lipoprotein-enriched fraction</td>
<td>5.9</td>
<td>79,397 ± 1,177</td>
</tr>
<tr>
<td>Nonlipoprotein fraction</td>
<td>36.6</td>
<td>43,518 ± 2,728 (43)</td>
</tr>
<tr>
<td>Normal human serum</td>
<td>58.8</td>
<td>95,559 ± 3,066</td>
</tr>
<tr>
<td>Lipoprotein-enriched fraction</td>
<td>7.7</td>
<td>63,182 ± 2,650 (10)</td>
</tr>
<tr>
<td>Nonlipoprotein fraction</td>
<td>56.5</td>
<td>89,100 ± 1,894</td>
</tr>
<tr>
<td>Control responses</td>
<td>76,169 ± 4,022</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte control (No PHA)</td>
<td>352 ± 200</td>
<td></td>
</tr>
</tbody>
</table>

Acid Ultrafiltration. To explore the possibility that a small molecule bound to a larger carrier molecule was responsible for the inhibition of lymphocyte function, SP II material was subjected to ultrafiltration under acid conditions. The purified SP II fraction containing lymphocyte-inhibitory activity was adjusted to pH 3.5 by addition of 0.01 M acetic acid:0.15 M NaCl buffer and subjected to ultrafiltration on a PM-30 membrane with four 5-fold dilutions of the retentate with the acetic acid solution. Material treated in this way did not reduce its capacity to inhibit the lymphocyte response to PHA, as noted by significant (p < 0.001) inhibition when tested in the assay (Table 4). Levels of inhibition were found to be comparable to that of the untreated material. After concentration to the original starting volume on a UM-05 membrane, the PM-30 filtrate was

Table 3
Rabbit anti-HSA affinity chromatography of electrophoretically purified lymphocyte-inhibitory activity

<table>
<thead>
<tr>
<th>Protein concentration (mg/ml)</th>
<th>Albumin concentration (mg/ml)</th>
<th>PHA response (cpm [³H] thymidine uptake)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP II-A</td>
<td>23.7</td>
<td>34.27</td>
<td></td>
</tr>
<tr>
<td>Anti-HSA effluent</td>
<td>4.0</td>
<td>3.60</td>
<td></td>
</tr>
<tr>
<td>Anti-HSA eluted</td>
<td>19.4</td>
<td>34.59</td>
<td></td>
</tr>
<tr>
<td>Control responses</td>
<td>48,515 ± 2,641</td>
<td>6,161</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte control (No PHA)</td>
<td>285 ± 51</td>
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<tr>
<td>Experiment B</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SP II-B</td>
<td>20.9</td>
<td>23.17</td>
<td></td>
</tr>
<tr>
<td>Anti-HSA effluent</td>
<td>4.4</td>
<td>4.58</td>
<td></td>
</tr>
<tr>
<td>Anti-HSA eluted</td>
<td>13.5</td>
<td>23.47</td>
<td></td>
</tr>
<tr>
<td>Control responses</td>
<td>34,258 ± 2,693</td>
<td>34,258 ± 2,683</td>
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</tr>
<tr>
<td>Lymphocyte control (No PHA)</td>
<td>377 ± 81</td>
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<td></td>
</tr>
</tbody>
</table>

Acid treatment and ultrafiltration of electrophoretically purified material containing lymphocyte-inhibitory activity

Electrophoretically purified material from Fraction D (SP II) was subjected to ultrafiltration under acid conditions (pH 3.5). After neutralization (pH 7.0) and concentration by Amicon ultrafiltration to the original sample volume, the samples were tested for their ability to inhibit the PHA response of normal lymphocytes. All fractions were tested at 40 μl/well.
observed to cause only slight inhibition (14%, $p < 0.05$) of the PHA response.

**Analytical Polyacrylamide Gel Electrophoresis of Fractions Containing Lymphocyte-Inhibitory Activity.** Whole clarified ascites fluid and purified fractions containing high levels of lymphocyte-inhibitory activity were subjected to analysis by electrophoresis on 6.5% polyacrylamide gels in a nondenaturing system. Gel scans of inhibitory fractions from a representative purification are illustrated in Chart 4. The electrophoretic profile of whole ascites fluid was similar to that of normal human serum. Gel scans of Fractions A and D revealed the fractions to be relatively enriched for proteins staining in the albumin and fast globulin regions, with a substantial amount of remaining proteins with low mobility on polyacrylamide gel electrophoresis. Electrophoretic analysis of the material contained in G-200 Pools IV and V of Fraction D demonstrates a substantial enrichment for proteins in the albumin and fast globulin region. Gel scans of SP II revealed the major proteins to be in the albumin region and 2 minor bands with RF's of 0.45 and 0.47. One of the minor bands was identified as albumin by Ouchterlony analysis with rabbit anti-HSA antisera and probably corresponded to the dimeric form of the molecule which often occurs upon concentration.

**SDS: Analytical Polyacrylamide Gel Electrophoresis.** SP II fractions from active ascites fluids, similarly purified fractions of noninhibitory ascites fluids and normal human serum, and the effluent and eluted fractions of the anti-HSA immunoabsorption column were analyzed by electrophoresis on 10% polyacrylamide gels containing SDS (data not shown). The major component detected appeared to have a mobility identical to serum albumin. Little difference could be detected among the different preparations.

**Analytical Isoelectric Focusing in Polyacrylamide Gels.** Electrophoretically purified material, SP II, and the respective eluted and effluent fractions from the anti-HSA immunoabsorption column were analyzed by analytical isoelectric focusing in polyacrylamide gels, and the results are shown in Chart 5. The material purified by semipreparative electrophoresis had a majority of stained protein in the pH 4.7 to 4.9 region corresponding to the isoelectric point of HSA. A minor series of 3 to 4 bands were detected in the pH 4.4 to 4.8 region, and a small amount of protein stained in the pH 3.9 region. SP II preparations of other inhibitory ascites fluids and of normal human serum which did not have lymphocyte-inhibitory activity revealed a very similar pattern upon analytical isoelectric focusing. The effluent fraction of the anti-HSA affinity column, which contained the inhibitory activity, revealed a major protein band in the pH 4.7 to 4.9 region and 6 to 7 protein bands in the pH 4.0 to 4.4 region and was found to be enriched for the proteins in the pH 3.9 region after removal of a substantial amount of albumin from the original SP II fraction. The anti-HSA column-eluted fraction demonstrated a major protein band in the pH 4.8 to 4.9 region.

**Immunological Analysis of Purified Fractions.** The SP II material and the anti-HSA immunoabsorbent effluent fractions were subjected to analyses by double diffusion in agar. Besides albumin, contaminating amounts of $\alpha_1$-antitrypsin and $\alpha_1$-acid glycoprotein were detected in both fractions. Levels of these 2 proteins were quantitated by radial immunodiffusion in purified material and the anti-HSA immunoadsorbent effluent fractions of other inhibitory ascites fluids and of normal human serum which did not have lymphocyte-inhibitory activity revealed a very similar pattern upon analytical isoelectric focusing. The effluent fraction of the anti-HSA affinity column, which contained the inhibitory activity, revealed a major protein band in the pH 4.7 to 4.9 region and 6 to 7 protein bands in the pH 4.0 to 4.4 region and was found to be enriched for the proteins in the pH 3.9 region after removal of a substantial amount of albumin from the original SP II fraction. The anti-HSA column-eluted fraction demonstrated a major protein band in the pH 4.8 to 4.9 region.

**Chart 4.** Nondenaturing polyacrylamide gel electrophoretic analysis of fractions containing lymphocyte-inhibitory activity sequentially followed through each purification step. Discontinuous polyacrylamide gel electrophoresis was carried out on 0.5- x 8-cm 6.5% polyacrylamide gels (200 $\mu$g of protein per gel) at room temperature for 3 to 4 hr at a constant current of 2.5 ma/gel. Gels were stained with Coomassie blue and scanned at 600 nm.

**Chart 5.** Analytical isoelectric focusing in polyacrylamide gels of SP II (A) and the effluent (B) and eluted (C) fractions from the anti-HSA immunoabsorbent column. Analytical isoelectric focusing was carried out in 0.5- x 10-cm 5% polyacrylamide gels with 2% ampholytes. Gels were preelectrophoresed to establish the pH gradient at 250 V for 45 min at 4$^\circ$ prior to application of the protein samples (100 $\mu$g of protein per gel). Electrophoresis was resumed for 6 to 8 hr at 350 V. Gels were stained with Coomassie blue and scanned at 600 nm. The pH gradient was determined by eluting the ampholytes from 0.5-cm gel sections for 1 hr with 1 to 2 ml of $\text{H}_2\text{O}$ and measuring the pH at 4$^\circ$. 

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The results of the present investigations demonstrate that the factor(s) in the ascites fluids described in these studies did not cause cancer patients to achieve recovery of the active moiety from the column. Since the lymphocyte-inhibitory activity resided in the lipoprotein fraction, we found to be separable from albumin by use of Affi-Gel Blue. The nature of this minor region of inhibitory activity is unknown at present, and the fact that the active dye:gel complex has been reported to have a very strong affinity for certain proteins (6).

Based on the findings of Glasgow et al. (17) and Nimberg et al. (33), we hypothesized that the active factor responsible for the inhibition of lymphocyte function in ascitic effusions may be a small molecule which binds to various proteins, as suggested for the lymphocyte-inhibitory peptide found in the serum of cancer patients. Recent evidence suggests that a small peptide with lymphocyte-inhibitory activity can be dissociated from carrier serum proteins by acid pH treatment (17, 46). We attempted to dissociate a small-molecular-size component with inhibitory activity from our purified preparations by acid ultrafiltration and were largely unsuccessful. However, an active, small-molecular-size component cannot be totally excluded, since the binding of such a component to a carrier molecule which copurified with albumin as described in these studies may be much stronger and may require more drastic conditions to effect dissociation.

The present investigations have also demonstrated that the lymphocyte-inhibitory activity could be separated into 2 different fractions upon preparative electrophoresis. Minor levels of activity were usually found with the proteins that eluted from the electrophoretically fast globulin (SP IV) region. The majority of the lymphocyte-inhibitory factor(s) in ascites fluids from ovarian cancer patients was found to copurify with albumin and probably accounted for the fact that relatively low specific activities and fold purifications were recorded. These results are consistent with the observations of Nimberg et al. (33), in which the majority of immunosuppressive activity in sera of cancer patients was shown to reside in a fraction which copurified with the albumins and β-globulins. Similar results were observed by Ting et al. (41), who showed that a humoral immunosuppressive factor in the ascitic fluid of mice bearing i.p. tumors and in pleural effusions from human cancer patients resided in a fraction which contained mostly albumin. The molecular weight of the immunosuppressive component in the studies described by Ting et al. (41) was less than 60,000, as estimated by gel filtration and SDS electrophoresis. In the present studies, the molecular size of the inhibitory factor associated with the ascitic effusions from ovarian cancer patients was estimated to be 40,000 to 80,000 daltons by Sephadex G-200 gel filtration. This size estimation assumes a globular shape for the inhibitory factor.

In contrast to these results are the findings of Badger et al. (2), who showed that an active moiety in human ascitic effusions was present in a large-molecular-size fraction from Sephadex G-200.

DISCUSSION

Recent studies have demonstrated that a substantial percentage of ascites fluids from ovarian cancer patients contains a nondialyzable, nontoxic factor(s) which is capable of inhibiting the blastogenic response of normal lymphocytes (19). The results of the present investigations demonstrate that the active factor(s) in several inhibitory ascites fluids from ovarian cancer patients could be partially purified by means of Con A-S-4B (negative) and DE52 ion-exchange chromatography with substantially high yields (68 to 74%). Although the inhibitory factor(s) in the ascites fluids described in these studies did not bind to the Con A-S-4B column, we cannot exclude the possibility that it is not a glycoprotein. The data suggest, however, that the active factor(s) does not have an exposed carbohydrate moiety recognized by the Con A molecule which is shared by many of the serum glycoproteins.

Analysis of purified fractions containing the lymphocyte-inhibitory activity by immunodiffusion and nondenaturing polyacrylamide gel electrophoresis revealed that the major protein in these fractions was albumin and probably accounted for the fact that relatively low specific activities and fold purifications were recorded. These results are consistent with the observations of Nimberg et al. (33), in which the majority of immunosuppressive activity in sera of cancer patients was shown to reside in a fraction which copurified with the albumins and β-globulins. Similar results were observed by Ting et al. (41), who showed that a humoral immunosuppressive factor in the ascitic fluid of mice bearing i.p. tumors and in pleural effusions from human cancer patients resided in a fraction which contained mostly albumin. The molecular weight of the immunosuppressive component in the studies described by Ting et al. (41) was less than 60,000, as estimated by gel filtration and SDS electrophoresis. In the present studies, the molecular size of the inhibitory factor associated with the ascitic effusions from ovarian cancer patients was estimated to be 40,000 to 80,000 daltons by Sephadex G-200 gel filtration. This size estimation assumes a globular shape for the inhibitory factor.
tein-poor fraction following fractionation by density gradient flotation, it did not appear to be an immunoregulatory lipoprotein as described by Curtiss and Edgington (12) which may have bound to the Affi-Gel Blue.

Ouchterlony analysis of purified preparations containing the active factor(s) revealed, in addition to the presence of albumin, contaminating amounts of $\alpha_1$-antitrypsin and $\alpha_1$-acid glycoprotein, both which have been shown to have immunosuppressive properties (1, 8). Levels of these 2 proteins in ascites fluids and purified fractions did not correlate with the presence of lymphocyte-inhibitory activity, suggesting that $\alpha_1$-antitrypsin and $\alpha_1$-acid glycoprotein do not account for the inhibitory activity described in the present studies. Similarly, $\alpha_2$-fetoprotein, a lymphoregulatory molecule which inhibits in vivo and in vitro lymphocyte function (31), was not found in the active ascites fluids and purified fractions containing lymphocyte-inhibitory activity. However, a molecular variant of any one of these proteins which may be highly immunosuppressive in very low levels and not discernible by antisera analysis cannot be ruled out.

Fractionation of 2 pools of normal human serum using Con A-S-4B and DE52 chromatography and preparative electrophoresis did not result in a fraction which contained inhibitory activity. However, the total absence of this lymphocyte-inhibitory factor(s) in normal serum cannot be excluded, since levels may be very low. The inability to measure inhibition of the lymphocyte response at low concentrations of the active moiety (19) may account for the serum results and the fact that only 40 to 45% of ascites fluids from ovarian cancer patients were found to contain the inhibitory activity (19). In support are the results from these investigations, which demonstrate that some inhibitory activity could be detected in a previously defined "negative" ascites fluid (no inhibitory activity when tested at the screening dose of 40 µl of whole fluid per well) after enrichment by partial purification and subsequent concentration.

Recent evidence has demonstrated the presence of an immunosuppressive factor(s) in the ascitic effusions from several patients with diseases other than cancer (2). In the present studies, lymphocyte-inhibitory activity in effusions from patients with benign and malignant neoplastic diseases not only shared the same in vitro immunosuppressive properties (19) but also was found to purify in a similar fashion. These data are consistent with the hypothesis of LeBien et al. (22) that the presence of a lymphocyte-antiproliferative factor(s) in ascitic effusions may constitute a host-mediated response which is not limited to malignant neoplasia. However, the frequency of ascitic fluids containing lymphocyte-inhibitory activity in cancer patients (2—4, 19) and in several murine tumor models (20, 27, 40—42, 46) 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 (15, 17, 25, 29).

In summary, lymphocyte-inhibitory activity in several ascitic effusions from patients with benign and malignant ovarian neoplastic diseases was found to copurify with albumin. The inhibitory factor was separable from the bulk of the albumin by means of a rabbit anti-HSA immunoadsorbent column. Analysis of the most purified fraction containing the inhibitory activity by isoelectric focusing in polyacrylamide gels revealed a somewhat heterogeneous population of proteins with respect to isoelectric point. Proteins in the pH 4.4 to 4.8 region (Chart 5) probably include $\alpha_1$-antitrypsin, since 2 to 3 isoelectric variants of this serum protein have been shown to be in this region (36). No apparent differences between active and inactive fractions could be demonstrated which may have been due to a masking effect of the albumin. Isoelectric focusing appeared to yield the greatest resolution of minor proteins in the purified fractions compared to SDS-polyacrylamide gel electrophoresis. Investigations are currently underway to purify the active factor(s) further by preparative isoelectric focusing. The origin of the inhibitory factor found in these ascitic effusions and its relationship to other serum and lymphoid cell-associated regulatory factors (21, 37, 44) remain to be defined.

REFERENCES


Partial Purification and Characterization of a Lymphocyte-inhibitory Factor(s) in Ascitic Fluids from Ovarian Cancer Patients

Allan D. Hess, Stanley A. Gall and Jeffrey R. Dawson


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