Differential Effects of an Immunosuppressive Fraction from Ascites Fluid of Patients with Ovarian Cancer on Spontaneous and Antibody-dependent Cytotoxicity

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ABSTRACT

The ascites fluids from humans with cancer that has metastasized to the peritoneum is suppressive of the immune response both in vitro and in vivo. The active moiety is present in a high-molecular-weight fraction which elutes in the void volume of a Sephadex G-200 column. This fraction (designated Peak I) has been shown to inhibit a number of in vitro responses and will inhibit the antibody response to sheep red blood cells in vivo. In this report, the Peak I protein fraction from the ascites of patients with ovarian cancer is shown to inhibit spontaneous cytotoxicity of human mononuclear cells against the myeloid cell line K562 and against the T-lymphoid cell line Molt-4F. This fraction was active at concentrations of 1 to 3 mg/ml. In contrast to this finding, it was not possible to demonstrate an inhibition of antibody-dependent cell cytotoxicity against chicken red blood cells. Preincubation of the effector cells with Peak I prior to addition to the chicken red blood cell targets or modification of the antibody concentration in the assay did not result in suppression; in fact, in many experiments, the Peak I potentiated cytotoxicity against chicken red blood cells. The Peak I proteins were also tested in an antibody-dependent cell cytotoxicity assay against a transformed cell line (HeLa cells), and there was no significant suppression of cytotoxicity. Peak I protein fractions prepared as controls from normal human serum and a congestive heart failure fluid were not suppressive, whereas the Peak I fraction from a cirrhotic fluid was suppressive of natural killing activity.

INTRODUCTION

Normal peripheral blood mononuclear cells are spontaneously cytotoxic to some transfused and tumor cells in vitro (14, 46, 54). This cytotoxicity or NK is now a well-recognized phenomenon that is particularly effective with human lymphocytes (41, 49) and has been described in mouse (15, 25) and rat (39) systems. NK takes place in the absence of any apparent antigenic stimulation. The possible significance of NK as a mechanism for immnosurveillance has generated much interest (22, 26, 27), and while the in vivo significance of NK in humans is not completely understood, there are several animal models where tumor resistance has been correlated to levels of NK (4, 13). A second in vitro cytotoxicity reaction is ADCC.

In ADCC, effector (killer) cells bearing receptors for the Fc portion of antibody (FcR) bind to and lyse antibody-coated target cells (36, 45). This mechanism of cell injury has been implicated in the immune response to a wide variety of tumors, allografts, and infectious agents in humans and experimental animals (5, 8, 11, 17, 47).

Our laboratory has carried out a number of studies on the immunosuppressive effects of ascites fluids from patients with cancer metastatic to the peritoneum. These fluids are inhibitory of a variety of immune responses both in vitro and in vivo. In this report, we have examined the effect of a high-molecular-weight fraction isolated from the ascitic fluids of 2 patients with ovarian cancer on the NK and ADCC activity of human peripheral mononuclear cells.

MATERIALS AND METHODS

Effector Cells. Mononuclear cells were isolated from heparinized human peripheral blood from normal healthy volunteers on Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) density gradients. The cells were washed 3 times in HBSS and resuspended at the desired concentration in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Flow Laboratories, Rockville, Md.) containing 10% heat-inactivated (56°, 30 min) fetal calf serum (Flow Laboratories), 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). This medium will be referred to as RPMI-10%.

Target Cells. The myeloid cell line K562 and the T-lymphoid cell line Molt-4F were maintained in stationary suspension in 75 sq cm plastic flasks in RPMI-10%. The tumor cells were passaged every 3 days and on the day preceding an assay. HeLa cells were also maintained in RPMI-10% and passaged by standard techniques every 3 to 4 days and on the day preceding an assay. CRBC were obtained from Grand Island Biological Co. (Grand Island, N. Y.) and were washed 2 times in 0.15 n NaCl and 2 times in HBSS prior to use.

51Cr Labeling of Target Cells. Approximately 5 x 10⁶ K562, Molt-4F, or HeLa cells (in suspension) and 10⁹ CRBC were resuspended in 1 ml RPMI-10%. One to 200 µCi of Na¹⁷⁵CrO₄ (specific activity, 200 to 500 Ci/g) (New England Nuclear, Boston, Mass.) were added to the target cells which were then incubated at 37° for 1 to 2 hr with occasional shaking. Care was taken to keep the HeLa cells in suspension. Labeled target cells were washed 3 times in HBSS, resuspended in RPMI-10%, and adjusted to the desired concentration.

Preparation of Ascitic Proteins. Sterile ascites fluids were obtained from 2 patients with ovarian cancer metastatic to the peritoneal cavity. These patients had not been treated with either chemotherapy or radiotherapy, and their ascites fluids...
have previously been shown to be immunosuppressive (1). These particular fluids were used for these experiments as they are currently being fractionated for purification of the immunosuppressive moiety (40). The fluids had a protein concentration of approximately 50 mg/ml and were dialyzed against phosphate-buffered saline (pH 7.4) and applied to Sephadex G-200 columns. The elution profile of a typical column run is shown in Chart 1. The high-molecular-weight fraction eluting first from the column (designated Peak I) has been shown to be immunosuppressive in a number of in vitro systems (1, 2, 40). This fraction was dialyzed against water and lyophilized and stored at −20°C. Prior to testing, the Peak I was solubilized in RPMI-10% and sterilized by Millipore filtration (Ha, 0.45 μm). We have performed this fractionation procedure with a number of different cancer ascites, and all Peak I fractions have been immunosuppressive. The same purification procedure was used to prepare Peak I fractions from normal human serum, the fluid from a patient with congestive heart failure, and the fluid from a patient with cirrhosis of the liver. The profile on Sephadex G-200 is similar to the cancer ascites in all cases, with some variation in the volumes of the different peaks. Three additional control protein samples were included in several of the experiments. These were freshly drawn normal human serum, Peak II proteins, and Peak III proteins which we have shown to be inactive as immunosuppressants in vitro (1, 2).

**Assay for NK Activity.** Effector cells were tested against 51Cr-labeled K562 and Molt-4F target cells. Triplicate cultures were established in round-bottomed microtiter plates in a total volume of 200 μl. Effector cells were added in 100 μl medium to give E:T cell ratios of 100:1, 50:1, 25:1, and 12.5:1. A constant number of 51Cr-labeled target cells (2 x 10⁴) were added to each well in 50 μl medium. The volume in each well was brought to 200 μl with RPMI-10%, Peak I, or control protein. The cultures were incubated at 37°C in 5% CO₂ incubator for 4 or 16 hr, and the supernatants were harvested using a Titertek supernatant collection system (Flow Laboratories). The amount of chromium released into the supernatant was determined in a Beckman 300 system gamma counter. Cytotoxicity was calculated as follows:

\[
\% \text{ of cytotoxicity} = \frac{\text{experimental } 51\text{Cr release} - \text{spontaneous } 51\text{Cr release}}{\text{total } 51\text{Cr counts} - \text{spontaneous } 51\text{Cr release}} \times 100
\]

In all experiments, spontaneous 51Cr release was measured with the target K562 and Molt-4F cells alone and target cells incubated with Peak I or control proteins. In no case did the added samples cause toxicity to the target cells. This absence of toxicity was shown by trypan blue exclusion and the lack of increased spontaneous release of radioactivity of the target cells.

**Assay for ADCC.** The ADCC assay against HeLa targets was established in a manner similar to that for NK against K562 in that similar numbers of effector and target cells were utilized. Antibody against HeLa cells was prepared in rabbits by repeated injections of 10⁷ to 10⁸ live HeLa cells i.p. Rabbits were bled by cardiac puncture, and the γ-globulin fraction of serum was prepared. Lyophilized γ-globulin was suspended in HBSS at a concentration of 4 mg/ml and titered to determine the concentration required for maximum killing (1:1000). Twenty-five μl of antisera were added to the cultures, and incubation was for 18 or 40 hr. The supernatants were harvested and counted as before, and the percentage of cytotoxicity was calculated.

ADCC activity of the mononuclear cells against CRBC was also determined. 51Cr-CRBC were adjusted to a concentration of 10⁷/ml, and 50 μl were pipetted into each well. Effector cells were added at 3 different concentrations giving E:T ratios of 4:1, 2:1, and 1:1. Anti-CRBC antibody was obtained from Cappel Laboratories, Inc. (Cochranville, Pa.), and 25 μl of 1:1000 dilution were added to the microtiter plates.

In all ADCC experiments, the background spontaneous cytotoxicity of the cells against CRBC and HeLa cell targets was examined in the absence of antibody. In addition, the Peak I and control proteins were incubated with target cells alone and with target cells and antibody. There was no additional spontaneous release of 51Cr label from the target cells due to antibody or sample addition.

**RESULTS**

The immunosuppressive activity of Peak I protein from an ovarian ascites fluid on NK against K562 targets in a 4-hr assay is shown in Chart 2. There was a dose-dependent suppression of NK activity at all 3 E:T cell ratios that were tested. The Peak I material was not cytotoxic to either the effector or the target cells. In the experiment shown in Table 1, the Peak II proteins were included as controls, and it can be seen that there was no suppression of the control response. The Peak I fraction (3 mg/ml) was suppressive at all E:T ratios tested. The Peak III proteins were included in many of the experiments and never showed any activity. Normal human serum and human serum albumin have also been used as controls in these experiments without demonstrating any immunosuppressive activity.

The immunosuppressive activity from a second Peak I fraction of ovarian ascites fluid is shown in Chart 3. This fraction was highly suppressive with 100% suppression being obtained with 1.25 mg protein per ml. In this experiment, we also tested the Peak I fractions from normal human serum, the fluid from a patient with congestive heart failure, and the fluid from a patient with cirrhosis of the liver. Various concentrations of the Peak I fractions were tested on NK activity at a 50:1 E:T ratio. High concentrations of the Peak I fractions from normal human serum and from the congestive heart failure fluid were required to give suppression of the response (approximately 5 mg
Modulation of NK and ADCC by Ascites Proteins

was no longer apparent at 40 hr, the effect of Peak I was examined in an 18-hr ADCC assay against CRBC. The results of this experiment are shown in Chart 4. It can be seen that the Peak I was still unable to suppress the ADCC; in fact, as noticed previously, these proteins enhanced the cytotoxicity for CRBC. This increase in killing was not due to toxic effects of Peak I on the target cells because the spontaneous release of $^{51}$Cr from labeled CRBC incubated with Peak I was no different to the release from CRBC incubated alone.

The absence of inhibition by Peak I in this assay could be due to some form of competition between the antibody and the immunosuppressive moiety for a site on the lymphocyte surface. Effector cells were therefore incubated with Peak I (3 mg/ml) in microtiter plates for 3 hr prior to the addition of antibody and CRBC. The cultures were then incubated for an additional 18 hr. As seen in Chart 5, despite the 3-hr preincubation of the effector cells with inhibitory material, there was still no evidence that the Peak I could inhibit the ADCC in an 18-hr assay. Cordier et al. (7) have demonstrated that high concentrations of $\alpha_2$-macroglobulin (a protein which is present in the Peak I fraction) can inhibit ADCC against CRBC and that this inhibition is markedly increased upon dilution of the anti-CRBC antibody. In order to test this in our system, the ADCC

Table 1

<table>
<thead>
<tr>
<th>E:T ratio</th>
<th>Control</th>
<th>Peak II (3 mg/ml)</th>
<th>Peak I (3 mg/ml)</th>
<th>% of suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:1</td>
<td>51.9 ± 5.1</td>
<td>56.9 ± 3.7</td>
<td>21.0 ± 2.9</td>
<td>60</td>
</tr>
<tr>
<td>50:1</td>
<td>52.7 ± 1.7</td>
<td>46.7 ± 3.0</td>
<td>22.3 ± 2.5</td>
<td>58</td>
</tr>
<tr>
<td>25:1</td>
<td>43.5 ± 5.4</td>
<td>43.0 ± 3.5</td>
<td>19.0 ± 3.4</td>
<td>56</td>
</tr>
</tbody>
</table>

% of suppression = 100 - (cytotoxicity of Peak I-treated cells / cytotoxicity of control cells) × 100

a Mean ± S.D.

b Treated cultures were significantly different from control cultures ($p < 0.01$).

c All Peak I-treated cultures were significantly different from control cultures ($p < 0.001$).

c ND, not determined.
Table 3
Effect of Peak I proteins on antibody-dependent cellular cytotoxicity of human mononuclear cells against 51Cr-CRBC
Cultures were incubated for 40 hr prior to harvesting. Statistical evaluations have not been included as there was clearly no immunosuppressive effect exerted by the Peak I proteins.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of cytotoxicity at following E:T ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4:1</td>
</tr>
<tr>
<td>Control untreated cultures</td>
<td>52</td>
</tr>
<tr>
<td>Peak I 3 mg/ml</td>
<td>61</td>
</tr>
<tr>
<td>1.5 mg/ml</td>
<td>59</td>
</tr>
<tr>
<td>0.75 mg/ml</td>
<td>59</td>
</tr>
</tbody>
</table>

Chart 4. Inability of Peak I proteins from ascites fluid (3 mg/ml) to inhibit ADCC of human mononuclear cells against antibody-coated CRBC target cells in an 18-hr assay. •, control untreated cultures; O, cultures containing Peak I proteins (3 mg/ml).

assay was established with 3 different concentrations of antibody: 1:1,000 (optimum); 1:10,000; and 1:50,000 (suboptimum). The Peak I proteins (3 mg/ml) were then tested for inhibitory activity at each antibody dilution. As can be seen in Chart 6, increasing dilutions of the antisera resulted in decreasing cytotoxicity against CRBC. There was, however, no evidence that Peak I proteins could inhibit the ADCC activity even at the lowest dilution of antibody.

ADCC activity of human peripheral mononuclear cells against an erythrocyte target (CRBC in this case) may not be identical to ADCC activity against a transformed cell line. There is evidence to suggest that different cells are involved. We have therefore established an ADCC assay against HeLa cells and have examined the effect of Peak I proteins in this assay. The results of one of these experiments is shown in Chart 7. Human mononuclear cells rarely display any natural cytotoxicity against HeLa cell targets. In one or 2 experiments, some activity could be detected, but it was minimal. In this experiment, no NK activity could be detected, but we were able to demonstrate good ADCC activity. As with the experiments utilizing CRBC as targets, we were unable to demonstrate any inhibition of the ADCC against HeLa cells with the Peak I proteins.

DISCUSSION

Human mononuclear cells are naturally cytotoxic for the myeloid cell line K562 and the T-lymphoid cell line Molt-4F. We have demonstrated that a high-molecular-weight fraction of human ovarian cancer ascites fluid (designated Peak I) which has previously been shown to be immunosuppressive (1, 2, 40) will inhibit this activity in a dose-dependent fashion. In direct contrast, the Peak I proteins are unable to inhibit ADCC activity of the same mononuclear cell fraction against CRBC or HeLa cell targets. In fact, in most of the experiments, we have found that these proteins will enhance cytotoxicity against CRBC in
the absence of toxicity. We do not have an explanation for this latter finding at this time, although it may be due to the presence of complement factors in the Peak I fraction. Ghebrehiwet and the absence of toxicity. We do not have an explanation for this factor is present in the Peak I fraction.

The differential sensitivity of these 2 cytotoxicity assays to the immunosuppressive activity of these Peak I proteins lends support to the hypothesis that there may be 2 different effector cells or 2 different effector mechanisms mediating cytotoxicity. There is currently a great deal of interest and controversy concerning the cells affecting NK and ADCC. NK cells have generally agreed that surface immunoglobulin-positive B-cells appear to have any surface immunoglobulin (52). It is therefore receptor-negative cells which may possess low-density or low-affinity receptors for sheep RBC (21, 57). They do not appear to have any surface immunoglobulin (52). It is therefore generally agreed that surface immunoglobulin-positive B-cells and macrophages are not effectors in NK. Several authors have identified the most active NK cell as a non-T-cell (20, 48); however, other workers claim that a T-cell may contribute to NK (3, 23), and others say that it is the main effector cell (18, 57). Koren and Williams (30) believe that NK and K-cells are 2 separate lymphoid populations, with NK cells bearing receptor sites for NK determinants and FcR, whereas K-cells bear only FcR. Insofar as the lymphocytes responsible for NK have in most instances been shown to have surface receptors for the Fc fragment of IgG, they are indistinguishable from the effector lymphocytes that mediate ADCC (24, 42, 43, 46). Many workers therefore consider that a significant fraction of both K- and NK cells are Fc receptor-bearing T-cells (43, 50, 56).

It would certainly appear from the experiments presented in this study that 2 different mechanisms are responsible for the cytotoxicity. It must be remembered, however, that in vitro experiments are always suspect of artifactual results. There are other studies which show that NK and ADCC have a differential sensitivity to certain substances such as interferon (9, 16, 34, 55), Protein A from *Staphylococcus aureus* (24), ouabain (32), enzymes (24), and corticosteroids (44). There are also studies which demonstrate differences in these 2 cytotoxic activities in various pathological states (12, 28, 29, 35, 51).

In addition to the controversy concerning the NK and ADCC cells, there is also controversy as to whether the cells mediating ADCC against CRBC are the same as the cells mediating cytotoxicity against transformed cell lines. Some researchers have demonstrated that monocytes, lymphocytes, and granulocytes are actively cytotoxic for CRBC (6, 38, 53), whereas a lymphocyte (K-cell) is active against transformed cells. In addition, Catalona et al. (6) have shown that, in cancer patients, ADCC against Chang cell targets is impaired, whereas the cytotoxicity against CRBC was normal. Due to these reports, we felt that it was necessary to examine the effect of the immunosuppressive fraction on a target other than CRBC. We established an assay with HeLa cells and found that mononuclear cells are spontaneously cytotoxic (NK activity) for HeLa cells, but poorly so. This NK activity, which ranged from 0 to 10%, was completely suppressed by the Peak I proteins at all E:T ratios. The Peak I proteins were not, however, able to significantly suppress the ADCC against the HeLa cell targets. In those experiments where some NK activity was evident, there was a minimal inhibition of the ADCC, usually not significant, which we hypothesize was a reduction of NK activity against the target cells which is taking place concomitantly with the ADCC.

There are some recent studies describing the immunosuppressive activity of serum and ascitic proteins on spontaneous cell cytotoxicity and ADCC. Cordier et al. (7) have described the inhibition of mitogen-induced cytotoxicity and ADCC against CRBC by a human α2-macroglobulin fraction. This inhibition apparently increases with dilution of the antisera in the assay. As there is an α2-macroglobulin in our Peak I preparation, we have tried to duplicate these findings with our immunosuppressive fraction without success. Kuo (31) has shown that EL4/C57Bl/6 ascitic fluid will block antibody complement-dependent cytolysis of murine spleen cells. This blocking, however, was reduced when excess complement was used in the assay and therefore does not appear to be similar to the system that we are concerned with. Nair et al. (37) have found that the sera from both normal and tumor-bearing animals inhibit the NK against RL5 1 and ADCC against CRBC, the cancer serum being the more inhibitory. We have found previously (1) that not only cancerous ascites are immunosuppressive but that fluids from patients with liver disease and cirrhosis suppress lymphocyte proliferation in response to phytohemagglutinin. We prepared Peak I fractions from a cirrhosis fluid and found that it suppressed NK activity of mononuclear cells against the K562 cell line. Peak I fractions from normal human serum and congestive heart failure were suppressive only at high concentrations (5 mg/ml). We did not test these fractions on ADCC function. Immunosuppressive activity in nonmalignant ascites has also been demonstrated by Hess et al. (19) and Le Bien et al. (33), and they suggest that these factors may be a natural host-mediated response to inflammation. It remains to be determined whether the suppressive moieties in these fluids are identical. Our current purification studies have centered on the ovarian ascites fluids and must be repeated with the cirrhosis and other inflammatory fluids.

Although we feel that the Peak I proteins have a direct
immunosuppressive effect on the mononuclear effector cells, we have not ruled out the possibility that this fraction may be activating a suppressor cell population. This suppressor cell population would have to be specific for the NK activity and not affect the ADCC activity. In addition, these suppressor cells would have to be activated very rapidly as the entire assay only takes 4 hr.

These studies clearly demonstrate that the spontaneous cytotoxicity of normal mononuclear cells is inhibited by ascitic fluid proteins, whereas their ADCC activity may not be compromised. This inhibition of NK activity may be a reflection of an in vivo reduction of the cancer patients' capability for immune surveillance.

REFERENCES


Modulation of NK and ADCC by Ascites Proteins


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