Expression of Cell-mediated Immunity and Blocking Factor Using a New Line of Ovarian Cancer Cells in Vitro

Roland A. Pattillo, Michael T. Story, and Anna C. F. Ruckert

Abstract

A cell-mediated cytotoxicity test, quantitated by postlabeling with tritiated thymidine, was used to assess immune reactivity of cancer patients to the HeW cell line derived from serous cystadenocarcinoma of the ovary. Lymphocytes from 71.4% of serous and mucinous cystadenocarcinoma patients demonstrated a cytotoxic response towards the HeW cells, whereas no reactivity was observed towards target cells derived from nonovarian cancer. These observations indicated that the HeW cells express tumor-associated antigen. In some patients bearing similar tumors, cytotoxicity was blocked by ascitic fluid from other patients with cystadenocarcinoma. In addition, antigen obtained from the spent culture fluid of HeW cells exhibited blocking activity in a typical dose-response fashion, suggesting that blocking factor may be free tumor-associated antigen or an antigen-specific suppressor molecule. Thus, blocking of the lymphocytotoxic response of cystadenocarcinoma patients towards HeW cells may be utilized to monitor the isolation of ovarian carcinoma-associated antigen.

Introduction

The high mortality rate among patients with epithelial carcinoma of the ovary has prompted a search for specific markers (TAA) for ovarian carcinoma. It is anticipated that assays for TAA would allow early diagnosis of ovarian cancer and provide a means of monitoring patient therapy in a manner analogous to that of the HCG marker associated with trophoblastic disease.

The approaches used in identifying ovarian TAA include: (a) in vitro tests for cell-mediated immunity including leukocyte migration (6, 26) and LMC (8, 9, 14); (b) production of xenogenic antisera to tumor extracts and demonstration of immune reactivity by immunoprecipitation and immunofluorescence (3-5, 11, 12, 22-25); and (c) isolation of TAA and associated antibody from immune complexes recovered from ascitic fluid (10).

Despite recent controversy regarding the immune specificity of LMC testing (30, 36, 39), immune reactivity to TAA in patients with ovarian carcinoma (8, 9, 14) and the observation that histologically similar tumors share common antigens (14) are supported by other experimental approaches. Chen et al. (6) reported that patients with serous and mucinous cystadenocarcinoma exhibited inhibition of leukocyte migration to autologous as well as homologous tissue antigens derived from these tumors. Their studies indicated that common antigens were present in serous and mucinous cystadenocarcinoma but not present in antigens derived from other gynecological neoplasms, including squamous cell carcinoma, leiomyosarcoma, and mixed mesodermal tumor of the uterus. Studies in which antisera were prepared against tumor extracts from patients with ovarian carcinoma (3-5, 11, 12, 22, 23) or from ovarian cancer cells in long-term culture (24, 25) leave little doubt that human ovarian cancer cells express unique antigens. Although the specificity of xenogenic antisera for ovarian carcinoma has not been established in all cases, at least one common antigen appears to be associated with serous and mucinous cystadenocarcinoma. The antigen appears to be a high-molecular-weight glycoprotein unrelated to blood group substances, carcinoembryonic antigen, α-fetoprotein, and histocompatibility antigen (4, 5). After appropriate absorption, the antiserum failed to react with normal ovarian tissue, benign ovarian cystadenoma, ovarian granuloma, and unrelated types of carcinoma (12, 22). It remains to be determined if xenogenic antiserum to ovarian carcinoma-derived antigen will be of value in early detection of neoplasia. In addition, experimental approaches utilizing antisera to tumor cells do not lend themselves to studies of blocking of lymphocytotoxicity by serum factors (13, 15, 27), which has been of interest as a possible explanation for in vivo escape by tumor cells from immune surveillance.

In this study, a recently established cell line (HeW) from serous cystadenocarcinoma of the ovary was used as a source of target cells in a LMC assay. The cytotoxicity response of lymphocytes from some patients was blocked by autologous serum and ascitic fluid from cystadenocarcinoma patients, as well as by antigens recovered from the culture fluid from HeW cells. These observations support the hypothesis that serum blocking activity may be due to free TAA or an antigen-specific suppressor molecule (29) and suggest that the blocking of cytotoxicity may be used to monitor the purification of TAA.

Materials and Methods

Establishment of the HeW Cell Line. HeW was derived from a 59-year-old Caucasian female who presented with a serious cystadenocarcinoma of the ovary widely metastatic to the peritoneal cavity in the presence of 12 liters of ascitic fluid. Within 72 hr after explantation of a solid tumor fragment in culture, migration of tumor cells from the primary explant was observed. Mechanical subculture was accomplished 16 days after explantation. Tumor cells were...
initiated in a medium consisting of 30% BSS, 50% Waymouth's 752/1, and 20% human umbilical cord serum (3520). The cells have been adapted to 3520 medium supplemented with 20% FCS and are now dispersed with 10× trypsin:EDTA. Flasks (25 sq cm; Falcon, Oxnard, Calif.) are subcultured at weekly intervals on a 1:2 split ratio. At present, the line has been sustained through 75 passages.

All tissue culture media, sera, and enzymes, with the exception of human umbilical cord serum, were obtained from Grand Island Biological Co., Grand Island, N. Y. Human umbilical cord serum was obtained after normal delivery from the obstetrical service at this institution.

Phase-contrast microscopic visualization of HeW cells (Fig. 1) reveals 2 major cell types. A population of large differentiated cells containing inclusion vacuoles staining positively for mucoprotein was seen as the predominant pattern in these cultures. The second cell type is represented by tightly packed islands and sheets of small angular cells with uniform nuclear size and distribution. Cells intermediate in the 2 morphological patterns described above suggest a progression from the smaller undifferentiated to larger differentiated cell type.

The cells are aneuploid with a modal number of 87 but with a wide distribution in chromosomal number about the mode (Chart 1).

The cells have the B-type G6PD isoenzyme pattern* and are free of Mycoplasma.

Radioimmunoassay and gel filtration analysis of the culture fluid from HeW cells for HCG and the α and β subunit, as previously described (2, 20), revealed low levels of material indistinguishable in size and immune reactivity from highly purified HCG β.5

Other cells used in this study to assess patient's cell-mediated immune response included the E1C0 (38), CaSki (34), and DoT (33) lines. The E1C0 line was derived from an American Indian female with infiltrative ductal adenocarcinoma of the breast and has been reported to have the A-type G6PD isoenzyme pattern and share karyological markers with HeLa (28). The CaSki and DoT lines were derived from epidermoid carcinoma of the cervix. CaSki cells have been reported to express TAA (34).

Patients. The cancer patients tested for LMC are shown in Table 1 under histological classification, FIGO staging of ovarian carcinoma, and treatment at the time of testing. Normal donor lymphocytes were obtained from healthy laboratory personnel.

Preparation of Lymphocyte Suspensions. Fifteen ml of venous blood, collected in preservative-free heparin, were diluted with an equal volume of sterile 0.9% sodium chloride solution and layered on a Ficoll-sodium metrizoate density gradient (specific gravity, 1.08) and centrifuged at 1200 rpm and at 4° for 40 min (Model PR-6; International Equipment Co., Needham Heights, Mass.). The lymphocyte-rich layer was recovered and washed 3 times with Roswell Park Memorial Institute Tissue Culture Medium 1640. Lymphocytes were counted with the aid of a hemocytometer, centrifuged, and brought to 2×10⁶ cells/ml in 3520 medium with 20% heat-inactivated FCS containing 10% human umbilical cord serum and 20% heat-inactivated FCS containing 10% human umbilical cord serum.

* W. A. Nelson-Rees, personal communication.
* R. O. Hussa, unpublished observations.
penicillin (50 units/ml) and streptomycin (50 µg/ml).

A second 7-ml sample of blood was collected without anticoagulant. The serum was recovered after centrifugation, heat inactivated, and added (20%) to 3500 medium for use in the blocking factor assay.

Ascitic fluid was aseptically obtained from a patient with cystadenocarcinoma and stored frozen at −70°C. After thawing, the fluid was clarified by centrifugation at 12,000 rpm and at 4°C for 40 min (Model RC-2B; Ivan Sorvall Inc., Norwalk, Conn.). The supernatant was filtered through Whatman No. 1 paper and sterilized by filtration (Millipore filtration, heat-inactivated, and added (20%) to 3500 medium for anticoagulant. The serum was recovered after centrifugation and at 4°C for 40 mm (Model L265B, SW 27 rotor; Beckman Instruments, Inc., Fullerton, Calif.). The supernatant was concentrated by molecular filtration (UM 20 membrane; Amicon Corp., Lexington, Mass.) and sterilized by filtration. Protein determinations, as described by Oyama and Eagle (31), were made on acid precipitates (32) dissolved in 1 N NaOH and neutralized with 1 N HCl.

RESULTS

Cell-mediated Reactivity. Table 2 presents data from incubations of ovarian cancer-derived cells (HeW) with lymphocytes from patients with ovarian cancer, other types of cancer, or healthy normal donors. In total, assays were performed with lymphocytes from 8 different normal donors, 21 patients diagnosed as having serous or mucinous cystadenocarcinoma, and 14 patients with other histological types of ovarian tumors, including endometrioid adenocarcinoma, poorly differentiated adenocarcinoma, clear cell adenocarcinoma, and benign cystadenoma. In addition, lymphocytes from 5 patients with cervical cancer and 2 patients with breast cancer were tested for cell-mediated reactivity.

The 8 normal donors were used as a source of control lymphocytes a total of 42 times. None showed significant cytotoxicity to the HeW cells. On the other hand, lymphocytes from 15 of 21 serous and mucinous cystadenocarcinoma patients showed cytotoxicity to the HeW cells. Of the 15 patients whose lymphocytes showed positive cell-mediated activity, 7 showed blocking of cytotoxicity when their lymphocytes were incubated with target cells in autologous serum-containing medium. As a test for possible nonspecific effects of autologous serum in the assay, the response of normal donor lymphocytes incubated in FCS-containing medium was compared with that of normal donor lymphocytes incubated in medium containing autologous serum. In 12 separate assays, no significant differences in normal donor lymphocyte response were observed (data not shown).

In incubations of lymphocytes from patients with other histological types of ovarian cancer (Table 2, Groups B to E), 6 of 14 differed from the response seen with normal donor lymphocytes. These included lymphocytes from 2 of 5 patients with endometrioid adenocarcinoma, 4 of 6 patients with poorly differentiated adenocarcinoma, 0 of 1 patient diagnosed as clear cell adenocarcinoma, and 0 of 2 patients with benign cystadenoma. None of these patients showed serum blocking activity. In addition, cytotoxicity but no serum blocking activity was observed with lymphocytes from only 1 of 5 patients with cervical carcinoma and 0 of 2 patients with breast carcinoma.

Lack of a Cell-mediated Reactivity of Serous or Mucinous Cystadenocarcinoma Patients to Nonovarian Carcinoma-derived Cells. Table 3 compares the response of serous or mucinous cystadenocarcinoma patient lymphocytes to the HeW cells and to 3 nonovarian cancer-derived cell lines established in our laboratory. Whereas lymphocytes from 15 of 21 serous or mucinous cystadenocarcinoma patients were cytotoxic to the HeW cells, lymphocytes of 6 days of maintaining cells on serum-free medium) was thawed, pooled, and centrifuged at 105,000 G and at 4°C for 90 min (Model L265B, SW 27 rotor; Beckman Instruments, Inc., Fullerton, Calif.). The supernatant was concentrated by molecular filtration (UM 20 membrane; Amicon Corp., Lexington, Mass.) and sterilized by filtration. Protein determinations, as described by Oyama and Eagle (31), were made on acid precipitates (32) dissolved in 1 N NaOH and neutralized with 1 N HCl.
from 0 of 9 patients and 0 of 8 patients were cytotoxic to the EICo and CaSki or DoT cells, respectively. Also shown in Table 3 is the response of normal donor lymphocytes to the nonovarian cancer-derived target cells. It is evident that normal donor lymphocytes frequently influenced tritiated thymide incorporation by the EICo, CaSki, and DoT cells. Nevertheless, lymphocytes from 10 of 10 different breast cancer patients showed a cytotoxic response which was significantly greater than was the influence of normal donor lymphocytes tested in the same assay. Similarly, lymphocytes from 4 of 4 different cervical cancer patients were cytotoxic to the epidermoid cervical cancer-derived cells (CaSki or DoT). On the other hand, the response of serous and mucinous cystadenocarcinoma patient lymphocytes to the nonovarian cancer-derived cells was no different from normal donor lymphocytes tested in the same assay. These
findings suggest that serous and mucinous cystadenocarcinoma patient lymphocytes are selectively cytotoxic to the HeW cells.

**Blocking Activity in Ascitic Fluid.** Since some of the cystadenocarcinoma patients demonstrated serum blocking activity (Table 2), it was of interest to determine if blocking activity was also present in ascitic fluid from patients with the same histological type of ovarian cancer. Lymphocytes from 3 different ovarian cancer patients were retested for cytotoxicity to the HeW cells and for blocking by ascitic fluid. Ascitic fluid was obtained from a patient (see Table 2, Group A, Patient 1) with advanced disease. The findings (Table 4) indicated that the cell-mediated cytotoxicity of all 3 patients was blocked by ascitic fluid (Chart 2).

Since ascitic fluid appeared to be a good source of blocking factor, it was of interest to determine the concentration of ascitic fluid required for blocking activity. The blocking activity of ascitic fluid from a patient with advanced serous cystadenocarcinoma was determined in an assay with lymphocytes from a patient with the same histological type of ovarian cancer. The dose-response curve of this blocking activity was seen at a concentration of approximately 0.7 μg of ascitic fluid protein per assay well. HeW cells incubated in ascitic fluid-supplemented medium (tested at the highest concentration used) incorporated 113.8 ± 17.8% (S.E.) of tritiated thymidine compared to 125.9 ± 18.8% in FCS-containing medium. These values were not significantly different, demonstrating that addition of ascitic fluid did not influence tritiated thymidine incorporation by HeW cells in the presence or absence of normal donor lymphocytes.

**Blocking Activity in a Crude HeW-derived Antigen Preparation.** Since soluble TAA may be responsible for blocking activity in patient serum and ascitic fluid, it was of interest to test an antigen preparation from HeW cells for possible blocking activity. A crude soluble antigen preparation was obtained from the cultur medium from HeW cells as indicated in "Materials and Methods." The preparation was found to have blocking activity when tested in 3 separate assays (data not shown). The dose-response of blocking activity in the crude HeW-derived preparation tested in an assay with lymphocytes from a cystadenocarcinoma patient (Table 2, Group A, Patient 16) is shown in Chart 3. A 50% reduction in blocking activity was seen at a concentration of approximately 0.12 μg protein per assay well.

The highest concentration of HeW antigen tested, 3.7 μg protein per assay well, appeared to be inhibitory to target cell growth. Target cells incubated in HeW antigen-supplemented medium incorporated 83.7 ± 12.1% of tritiated thymidine compared to control cells receiving FCS-containing medium. Target cells incubated with normal donor lymphocytes in HeW antigen-containing medium incorporated 60.2 ± 15.3% of label compared to 95.9 ± 11.2% in FCS-containing medium. Thus, the lower blocking index compared to 125.9 ± 18.8% in FCS-containing medium.

**Table 3**

<table>
<thead>
<tr>
<th>Cancer type or normal donors</th>
<th>Cell line</th>
<th>LMC response of cancer patients' and normal donors' lymphocytes to ovarian cancer and nonovarian cancer-derived cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum or mucinous cystadenocarcinomas</td>
<td>HeW 15/21</td>
<td>E1Co 0/8 CaSki or DoT 0/8</td>
</tr>
<tr>
<td>Breast carcinomas</td>
<td>0/2</td>
<td>10/10</td>
</tr>
<tr>
<td>Cervical carcinomas</td>
<td>1/5</td>
<td>1/7</td>
</tr>
<tr>
<td>Normal donors</td>
<td>0/8</td>
<td>15/21</td>
</tr>
</tbody>
</table>

a Number of patients whose response differed significantly from that of normal donors per number of patients tested.

b Number of normal donors whose response differed significantly from target cells incubated without lymphocytes per number of normal donors tested.

**Chart 2.** Dose response of ascitic fluid blocking activity. Values are the average of duplicate samples. Vertical bar, range.

**Table 4**

<table>
<thead>
<tr>
<th>Patients</th>
<th>% Lymphocytes incubated in FCS-containing medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No lymphocytes + ascitic fluid</td>
</tr>
<tr>
<td>Group</td>
<td>No.</td>
</tr>
<tr>
<td>A</td>
<td>3</td>
</tr>
<tr>
<td>A</td>
<td>7</td>
</tr>
<tr>
<td>A</td>
<td>11</td>
</tr>
</tbody>
</table>

a See Table 2.
b Mean ± S.E.
c Not significantly different from target cells incubated in the absence of lymphocytes in FCS-containing medium.
d Differs (p < 0.05) from control lymphocytes incubated in FCS-containing medium.
e Differs (p < 0.05) from patient lymphocytes incubated in FCS-containing medium.
f Not significantly different from control lymphocytes incubated in FCS-containing medium.
seen when target cells are incubated with patient lymphocytes in the presence of 3.7 μg HeW protein-containing medium (Chart 3) appears to be a result of inhibition of target cell growth.

**DISCUSSION**

Cell-mediated immune testing of patients with cancer has provided information suggesting that their lymphocytes exert a cytotoxic response to autologous tumor cells or to tumor cells of the same histological type (13-15) and that factors are present in patient serum which modify lymphocyte reactivity (13, 15, 27). However, much of what was thought to be reactivity to TAA may really be due to a reaction described as natural cytotoxicity (30, 36, 39). We have also observed lymphocyte reactivity in normal donors and in some cancer patients which is best described as natural cytotoxicity (Table 3). However, the observation that lymphocytes from 71.4% of serous or mucinous cystadenocarcinoma patients demonstrated cytotoxicity to the ovarian cancer-derived cells (Table 2, HeW) is not explained by the phenomenon of natural cytotoxicity since lymphocytes from normal donors and from 6 of 7 nonovarian cancer patients failed to show reactivity to the HeW cells. Furthermore, this explanation appears inconsistent with the observation that lymphocytes from cystadenocarcinoma patients showed no reactivity towards nonovarian carcinoma-derived cells above the response of normal donor lymphocytes tested in the same assay (Table 3). On the other hand, lymphocytes from breast and cervical cancer patients were reactive towards ElCo and CaSki or DoT cells, respectively. These observations suggest that lymphocytes from cystadenocarcinoma patients show selective cytotoxicity towards ovarian carcinoma-derived cells and, therefore, that the HeW cells express TAA.

Although in the present study no attempt was made to correlate patient lymphocyte reactivity or serum blocking activity with such factors as stage of disease or treatment modality, other investigators (16, 27, 37) have attempted to correlate in vitro immune response to TAA during therapy and clinical status. However, because of the technical difficulties encountered in microcytotoxicity testing, it has been difficult to determine whether the differences in patient responses were due to biological variations or to day-to-day changes in assay conditions (2, 19). Because of the possible significance of blocking factor in escape by tumor cells from immune surveillance (7, 17, 18) and the inherent difficulty in interpreting LMC data from individual patients, it is essential that other approaches be found for assessing serum blocking activity.

Regardless of the nature of blocking factor (17, 18, 29), in order to pursue this objective it is necessary to (a) determine if blocking factor from one patient is immunologically similar to that found in another patient with the same type of disease, (b) identify a good source of material with blocking activity, and (c) have a reliable biological endpoint for monitoring the isolation of blocking factor. The present study in part fulfills these requirements. Ascitic fluid exhibited blocking activity when tested against allogeneic lymphocytes (Table 4), suggesting that cystadenocarcinoma patients have blocking factor which is immunologically similar or identical. The titration of ascitic fluid from a patient with cystadenocarcinoma for blocking activity (Chart 2) suggests that blocking factor in this fluid is present in high amounts or is active in very low concentrations. Although ascitic fluid appears to be a good source of blocking factor, it is perhaps more significant that concentrates of the culture fluid from HeW cells blocked the cytotoxic response of lymphocytes from cystadenocarcinoma patients (Chart 3). The observation appears consistent with the hypothesis that blocking factor may be free TAA or an antigen-specific suppressor molecule (29) and supports earlier reports that TAA is shed by tumor cells in culture (1, 34, 35). Finally, it is anticipated that the isolation of blocking factor will be facilitated by using spent culture fluid as a source of material rather than serum or ascitic fluid since cell culture material represents a standard source of antigen in contrast to the heterogeneity of patient material. Efforts in this laboratory are currently directed towards identifying common antigens shared by the HeW cells and serum and ascitic fluid from cystadenocarcinoma patients.

**ACKNOWLEDGMENTS**

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**REFERENCES**

Fig. 1. Phase-contrast photomicrograph of HeW cells. × 100.
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