Reactivity of a Monoclonal Antibody with Human Ovarian Carcinoma

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ABSTRACT A murine monoclonal antibody (OC125) has been developed that reacts with each of six epithelial ovarian carcinoma cell lines and with cryopreserved tumor tissue from 12 of 20 ovarian cancer patients. By contrast, the antibody does not bind to a variety of nonmalignant tissues, including adult and fetal ovary. OC125 reacts with only 1 of 14 cell lines derived from nonovarian neoplasms and has failed to react with cryostat sections from 12 nonovarian carcinomas.

INTRODUCTION

Ovarian carcinoma cells exhibit distinct cell surface antigens that distinguish them from cells of normal adult tissues including ovary (1–7). Tumor-associated antigens (TAA)[1] have provided promising targets for immunodetection and immunotherapy of ovarian cancer in clinical studies and in animal models (8–11). Circulating TAA have been found in the blood of some ovarian cancer patients at a time when their tumors were still potentially curable by surgery alone (12). More advanced disease can respond dramatically to cytoreductive surgery and chemotherapy, but even following a complete clinical response the tumor generally recurs within 2–5 yr (13, 14). In this setting, immunotherapy might eliminate the relatively small number of tumor cells that remain after more conventional treatment. Clinical trials to date have used active immunotherapy with immunostimulants and tumor cell vaccines (15–19). Work in animal systems suggests, however, that ovarian carcinoma may be one of the tumors that will respond to treatment with antisera directed against TAA (8, 9, 11).

In the past, heteroantisera against human TAA have usually required extensive absorbtion to remove activity against nonmalignant tissues. Absorbed antisera are often of low titer and specificity has varied between different preparations. With the development of somatic cell hybridization techniques, large quantities of high titered monoclonal antibody can now be produced that does not require absorption (20). Moreover, each hybridoma produces a chemically homogeneous immunoglobulin that permits the selection of antibodies of a particular functional subclass as well as of a unique specificity. During our earlier studies, conventional rabbit heteroantisera were prepared against human and murine ovarian carcinomas (7–9, 21). In our report a murine monoclonal IgG1 immunoglobulin has been developed with specificity for human ovarian carcinoma.

METHODS

Human tissues. Ascites fluid from patients with ovarian carcinoma was obtained at the time of therapeutic paracentesis, ovarian tumor tissue was obtained at surgery, and portions of nonmalignant tissue were obtained after surgical resection or at autopsy performed within 6–12 h of death, using protocols approved by the appropriate Human Protection Committees. Tumor cells were separated from leukocytes and erythrocytes in ascites fluid using discontinuous gradients of Ficoll-Hypaque or bovine serum albumin (21). Solid ovarian tumor and benign ovarian tissue were minced into 1-mm^3 fragments and dissociated enzymatically by incubating for periods of 1–24 h in 0.1% collagenase III (Worthington Biochemical Corp., Freehold, N. J.) in Hanks' balanced salt solution (HBSS), pH 7.0. Ascites tumor cells and dissociated ovarian tissues were washed twice in Eagle's minimum essential medium (MEM, Grand Island Biological Co., Grand Island, N. Y.) and supplemented with 5% fetal bovine serum. Washed, dissociated cells were then cryopreserved in medium that contained 10% FBS and 10% dimethylsulfoxide and stored in liquid nitrogen.

For preparation of cryostat sections, small blocks of malignant or nonmalignant tissue were immersed in Tissue-Tek embedding medium (Lab-Tek Div., Miles Laboratories, Inc., Naperville, Ill.) and frozen with dichlorodifluoromethane

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[1] *Abbreviations used in this paper*: CEA, carcinoembryonic antigen; sIg+, surface immunoglobulin-positive; sIg-, surface immunoglobulin-negative; TAA, tumor-associated antigens.

(Cryokwik, Damon Corp., I.E.C. Div., Needham Heights, Mass.). Blocks were stored at −80°C until use.

Nucleated bone marrow cells were recovered by Ficoll-Hypaque centrifugation (22). Tonsil cells were obtained at the time of routine tonsillectomy. Lymph node tissue was obtained at diagnostic biopsy, and was considered normal based on histology and cell surface markers. All tissue specimens were immediately placed in media containing 5% fetal bovine serum, finely minced with forceps and scissors, and subsequently made into a single cell suspension by being pressed through stainless steel mesh.

Isolation of lymphocyte subpopulations. Human peripheral blood mononuclear cells were isolated from healthy volunteer donors by Ficoll-Hypaque density gradient centrifugation (22). Unfractionated mononuclear cells were separated into surface immunoglobulin-positive (sIg+) and surface immunoglobulin-negative (sIg−) populations by Sephadex G-200 anti-F(ab)$_2$ chromatography (23) with modifications designed to minimize monocyte retention by the column (24). Peripheral blood mononuclear cells were preincubated at 37°C for 1 h to remove cytophilically-bound serum IgG (25), and cells were applied to the anti-F(ab)$_2$ column and fractionated as outlined (23). The sIg+ (B) population was obtained from the Sephadex G-200 column by competitive elution with normal human gamma globulin. B cell preparations were routinely >90% sIg+, <5% sheep erythrocyte rosette-positive (E+), and contained ~5% monocytes as judged by morphology and latex ingestion. T cells were recovered by E rosetting the sIg- population with 5% sheep erythrocytes (Microbiological Associates, Bethesda, Md.). The rosetted mixture was layered over Ficoll-Hypaque and the recovered E+ pellet was treated with 0.155M NH_4Cl to lyse erythrocytes. The T cell population obtained was <2% sIg+ by described methods (26). Normal human monocytes were obtained by adherence to plastic culture dishes as described (27).

Cell lines. Six cell lines have been established from different ovarian carcinomas.[2] Each of the cell lines exhibited a distinctive epithelial morphology, karyotype, and pattern of growth in vitro. Before use, cells were detached from plastic substrates using trypsin-EDTA (Microbiological Associates).

In addition to the ovarian carcinoma cell lines, a cell line has also been established from a nonmalignant ovary which has maintained epithelial morphology.[3] This cell line has a population doubling of 24 h and a hypodiploid karyotype.

In four instances it has been possible to establish Epstein-Barr transformed B lymphocyte cell lines from the peripheral blood leukocytes of the patients from whom tumor cell lines had been established (28). The B lymphocyte lines have provided a source of species-specific antigens as well as autologous HLA and Ia antigens. Other cell lines used are listed in Table I.

Preparation and screening of hybridomas. To produce monoclonal immunoglobulins with specificity for human ovarian carcinoma, BALB/c mice were immunized with an epithelial cell line OVCA433 established from a patient (E.M.) with serous papillary cystadenocarcinoma. 10^6 viable tumor cells were injected intraperitonally on two occasions. 1 wk after the second injection 10^6 cells were injected intravenously and 3 d thereafter immune spleen cells were fused with the P3/NS-1 plasmacytoma line using 30% polyethylene glycol according to Kennet's modification (29) of the technique of Kohler and Milstein (20). Hypoxanthine, aminopterin, and thymidine were added 24 h later and colonies appeared in 10–28 d.

TABLE I
Reactivity of OC125 with Tumor Cell Lines

Line	Source	Binding of OC125
OVCA433	Ovarian carcinoma	++++
OVCA429	Ovarian carcinoma	++++
OVCA432	Ovarian carcinoma	++++
OVCA424	Ovarian carcinoma	++++
OVCA420	Ovarian carcinoma	++
OVCA400	Ovarian carcinoma	++++
CX-1	Colon carcinoma	−
SK-N-MC	Neuroblastoma	−
LX-1	Oat cell carcinoma	−
HeLa	Cervical carcinoma	−
MEL394	Melanoma	++
MEL388	Melanoma	−
MEL382	Melanoma	−
MEL368	Melanoma	−
W402	Pancreatic carcinoma	−
W404	Renal cell carcinoma	−
B734	Breast carcinoma	−
CEM	T Cell ALL	−
LAZ221	Non-T Cell ALL	−
K562	Chronic myelogenous leukemia	−

To determine specificity, supernates from cultures that contained colonies were screened using indirect immunofluorescence (30) analyzed by flow cytometry either on the FACS-1 (Becton, Dickinson & Co., Rutherford, N. J.) or on a cytofluorograph FC200/4800A (Ortho Instruments, Westwood, Mass.). Clones were sought that reacted with human ovarian carcinoma cell lines but failed to react with an autologous B cell line (LAZ 444) or with enzymatically dissociated cells from an allogeneic normal ovary. Among 166 wells with colonies screened, 4 reacted with the tumor cell line OVCA 433. Of these, two failed to react with the autologous B cell line, LAZ 444. Colonies forming antibody of appropriate specificity were cloned by limiting dilution on monolayers of irradiated BALB/c spleen cells and passaged as ascites in mice primed by the injection of 0.5 ml i.p. pristane 14 d before hybridoma injection.

Indirect immunofluorescence with single cell suspensions. Reactivity with monoclonal antibody was evaluated by indirect immunofluorescence as described (30). In brief, 1–2 × 10^6 cells were incubated with media-containing antibody for 30 min on ice, washed twice with phosphate-buffered saline (pH 7.4), and stained with a combination of fluoresceinated goat antimouse IgG and antimouse IgM (G/M FITC) (Meloy Laboratories, Springfield, Va.) for 30 min on ice. After two additional washes, fluorescent antibody-coated cells were analyzed by flow cytometry. Intensity of fluorescence was determined for 40,000 cells in each population and compared with the fluorescence of nonreactive antibody of identical isotype. With ascites populations the machines were gated to exclude small inflammatory cells. A displacement of the histogram of the test monoclonal antibody from that of the unreactive monoclonal antibody was scored as positive. In addition, for each test sample, a quantitative assessment of the number of positive cells was made. For analysis, cells

[2] Lazarus H., B. Malone, R. C. Bast, Jr., and R. C. Knapp. Manuscript in preparation.

[3] Lazarus H., B. Malone, R. C. Bast, Jr., and R. C. Knapp. Manuscript in preparation.

were considered positive that fell outside channels that contained the major peak of activity of control cells incubated with a nonreactive murine antibody and fluoresceinated goat antimouse immunoglobulin. A difference of >10% between experimental and control values was required to attain significance. Positive patterns were graded 1–4 plus based upon the degree of difference from control patterns. In all experiments, hybridoma ascites fluid containing monoclonal immunoglobulin was used as the source of antibody. The subclass of the immunoglobulin that bound to tumor cells was determined by indirect immunofluorescence after incubation with fluorescein-conjugated goat antimouse IgG1, IgG2 and IgM (Meloy Laboratories Inc., Springfield, Va.).

To evaluate the phenotype of cells isolated from peripheral blood and normal lymphoid tissues a panel of specific monoclonal antisera were used. Anti-T3 (OKT3) has been shown to react specifically with 100% peripheral blood T cells (31). The B cell fraction was enumerated by a mixture of antisera directed against the immunoglobulin light (κ and λ) chains. These reagents were kindly provided by Dr. Victor Raso, Sidney Farber Cancer Institute, Boston, Mass.

The presence of the HLA-D/DR-related Ia-like antigens was evaluated with a monoclonal antibody (I-2) (32) defining a nonpolymorphic antigen and precipitating the bimolecular complex 29,000 and 34,000. The Bl antigen has been shown to react specifically with all peripheral blood B cells and B cells from all lymphoid tissues excluding plasma cells and is unreactive with cells of any other lineage (33).

Indirect immunofluorescence with tissue sections. Reactivity of monoclonal antibodies with cryostat sections of malignant and nonmalignant tissue was analyzed as described previously (34, 35). In brief, four micron sections were incubated for 30 min at ambient temperature with 1:5, 1:10, and 1:100 dilutions of OC125 ascites fluid or with nonreactive Cl 287, an IgG1 antihuman IgM as a control. After washing three times for 10 min in PBS, sections were incubated with a 1:10 dilution of fluoresceinated rabbit antimouse immunoglobulin in (N. L. Cappel Laboratories, Inc., Cochraneville, Pa.) that had been absorbed with acetone-fixed human liver or kidney powder and with human serum that had been coupled to Sepharose with cyanogen bromide. After further washing, sections were examined with a fluorescence microscope equipped with epiillumination and a phase optics (Carl Zeiss, Inc., New York) (36).

RESULTS

The most promising clone isolated to date, designated OC125, produces an IgG1 immunoglobulin which binds to 6 of 6 epithelial ovarian carcinoma cell lines (Table I) and to cryopreserved tumor cells from ascites fluid in 7 of 11 ovarian cancer patients. OC125 reacts with only 1 of 14 tumor cell lines established from a variety of nonovarian neoplasms (Table I). The one nonovarian tumor cell line to which OC125 binds, MEL394, was derived from a melanoma. The antibody did not, however, bind to three other melanoma lines.

Extensive studies have failed to demonstrate binding of OC125 to nonmalignant tissues. The OC125 immunoglobulin was selected originally for reactivity with the OVCA433 ovarian tumor cell line and for lack of reactivity with a B lymphocyte line (LAZ 444) established from the same individual (Fig. 1). Binding of OC125 to the surface of the OVCA433 tumor cell line can be detected at dilutions of 1:1,000 to 1:10,000 of ascites fluid using indirect immunofluorescence. A lack of reactivity with autologous B lymphocytes at a dilution of 1:10 suggests that OC125 does not recognize HLA or Ia-like antigens known to be present on this line.

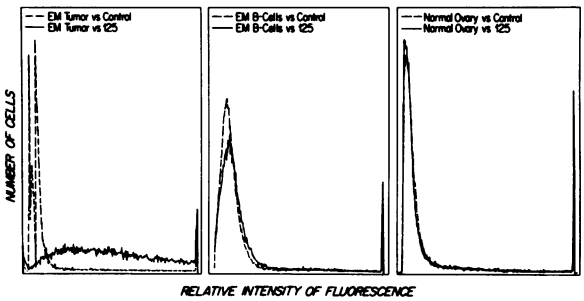

FIGURE 1 Reactivity of monoclonal antibody OC125 with 1) an epithelial ovarian carcinoma cell line OVCA433 against which the antibody had been raised (EM Tumor), 2) an Epstein-Barr virus-transformed lymphoblastoid cell line from the same individual (EM B cells) and 3) enzymatically dissociated nonmalignant allogeneic ovarian tissue (normal ovary).

A more rigorous test for reactivity with components, which might be present in trace amounts on the B cell surface, has been sought by quantitative absorption of antibody activity. A dilution of OC125 was found that could place 70% of OVCA433 cells outside the control peak for fluorescence intensity. Four absorptions with 10^7 OVCA433 tumor cells virtually removed antitumor reactivity, whereas absorption with 10 times as many LAZ 444 B cells failed to affect binding of the OC125 to OVCA 433 cells (Table II).

By flow cytometer analysis, OC125 does not react with enzymatically dissociated cells from adult or fetal ovary and does not bind to a cell line established from nonmalignant adult ovary. The majority of malignant ovarian tumors that develop in the adult, however, are thought to arise from the germinal epithelium that covers the ovarian surface and lines subcapsular cysts. OC125 might react with a normal differentiation antigen that is associated with the surface epithelium,

TABLE II
Reactivity of OC125 with OVCA433 Cells before and after Absorption

Antiserum	Absorption	Cells positive
		%
CONTROL	—	1.6
OC125	—	72.9
OC125	OVCA433 Tumor cells 4×10^7	5.0
OC125	LAZ444 B Cells 4×10^8	73.3

and this subpopulation of cells would be diluted with other cell types during dissociation of the ovary. To test the possibility that OC125 might bind to a subpopulation of nonmalignant ovarian cells, cryostat sections of seven adult ovaries were examined by indirect immunofluorescence after incubation with OC125 and fluoresceinated rabbit antimouse immunoglobulin. No significant fluorescence was associated with the surface epithelium or with other structures in adult ovaries (Fig. 2A, B). By contrast, bright fluorescence was associated with the epithelial component in sections of ovarian carcinomas from five of nine patients (Fig. 3A). The brightest staining was on the luminal surface of the malignant epithelium, which formed papillary projections and invasive glandlike structures (Fig. 4A, B). When control sections were incubated with a murine antihuman IgM monoclonal immunoglobulin of the same subclass (clone 287), staining with fluoresceinated goat antimouse immunoglobulin failed to produce significant fluorescence (Fig. 3B).

Because OC125 binds to antigen with sufficient avidity to permit examination of tissue sections by indirect immunofluorescence, we have examined the reactivity of the antibody with cryostat sections from a number of normal adult tissues (Table III). No reactivity could be detected with any of the normal tissues tested including uterus, breast, fallopian tube, skin, lung, liver, spleen, or kidney, nor did OC125 stain any of 12 carcinomas from other sites (Table III). In addition OC125 has failed to react with a variety of hematopoietic and lymphoreticular tissues which can be analyzed in suspension by indirect immunofluorescence using the flow cytometer, including allogeneic T cells, B cells, null cells, monocytes, AB erythrocytes, bone marrow, tonsil, and lymph node cells (Table IV).

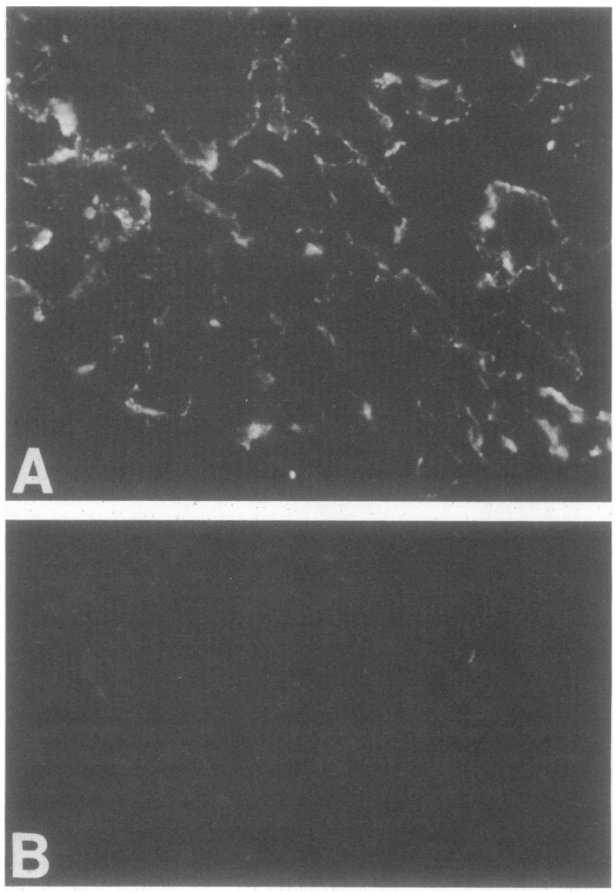

FIGURE 3 Immunofluorescence photomicrographs of cryostat sections of an ovarian papillary serous adenocarcinoma. (A) stained with OC125, (B) stained with control C287 (antihuman IgM). Bright surface staining of cells in papillary projections is seen in (A) but not (B). The stroma does not stain. ×320.

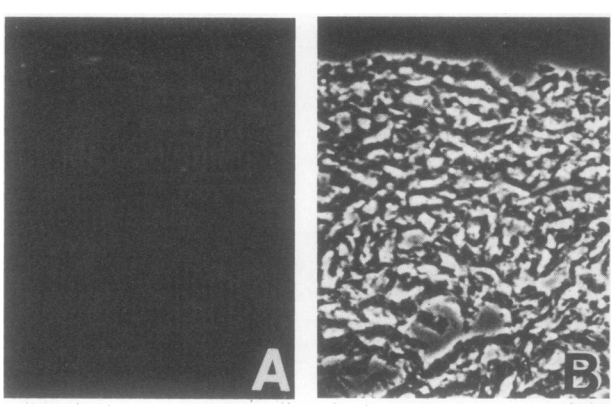

FIGURE 2 (A) Photomicrograph of a cryostat section of a normal adult ovary stained with OC125 and fluorescein-conjugated antimouse IgG. No appreciable staining is detected. (B) Same field, phase contrast with transmitted light shows that the surface epithelium is present. ×230.

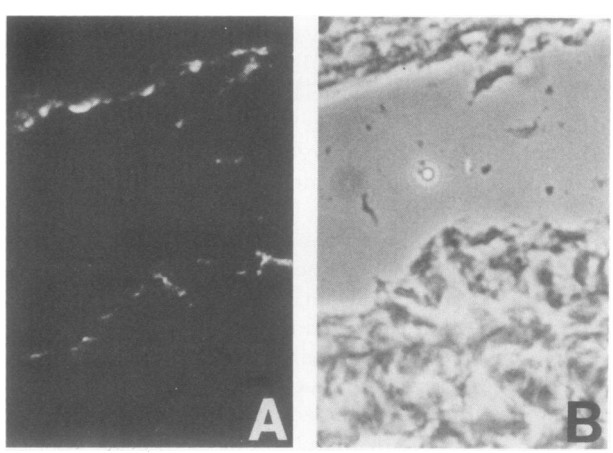

FIGURE 4 Same case as Fig. 3. (A) Malignant glands invading stroma show bright fluorescence staining with OC125 along the luminal surface (B), phase contrast same field. ×510.

TABLE III
Specificity of OC125 by Indirect Immunofluorescence in Cryostat Tissue Sections

Carcinomas	
Ovary	5/9
Other primary sites	0/12*
Normal tissue	
Ovary	0/7
Other normal tissue	0/26‡

* Includes samples of carcinoma of breast (4), kidney (3), colon (2), lung (1), uterus (1), and testes (1).
‡ Includes samples of uterine corpus (3) and cervix (3), fallopian tubes (2), breast (2), kidney (4, one fetal), lung (2), liver (3), spleen (3), and skin (3).

To test the possibility that OC125 detects carcinoembryonic antigen (CEA), the specific OC125 antibody or the irrelevant clone 287 was incubated with ^{125}I-labeled CEA. Immunoglobulin was precipitated with zirconyl phosphate gel. No more labeled CEA was coprecipitated with OC125 than with a nonspecific monoclonal reagent (Table V). Similar numbers of counts were precipitated by zirconyl phosphate gel in the absence of immunoglobulin. Labeled CEA did coprecipitate, as expected, in the presence of specific heteroantiserum, and precipitation of labeled CEA could be inhibited with nonlabeled CEA (Table V).

TABLE V
*Coprecipitation of ^{125}I-labeled Carcinoembryonic Antigen (^{125}I-CEA) with Monoclonal Antibodies (OC125, 287) or Specific Heteroantiserum (anti-CEA) in the Presence of Zirconyl Phosphate Gel**

Reagents	mean cpm in precipitate
^{125}I-CEA + diluent	8,112
^{125}I-CEA + OC125 (1:500)	8,002
^{125}I-CEA + OC125 (1:100)	8,052
^{125}I-CEA + CL287 (1:500)	7,971
^{125}I-CEA + anti-CEA	21,981
^{125}I-CEA + anti-CEA	
+ 1.2 ng CEA	27,287
+ 3.1 ng CEA	21,487
+ 6.2 ng CEA	17,686
+ 12.5 ng CEA	13,034

* Antibodies and nonlabeled CEA (Hoffman-LaRoche, Inc., Nutley, N. J.) were diluted in 0.01 M ammonium acetate buffer, pH 6.5 and incubated with 110,000 cpm ^{125}I-CEA for 30 min at 45°C. Free immunoglobulin and immune complexes were precipitated with 50% saturated zirconyl phosphate gel. Each precipitate was washed once with 0.1 M ammonium acetate buffer, dried, and counted. The ability of zirconyl phosphate gel to precipitate OC125 activity was confirmed by indirect immunofluorescence. OC125 activity was assayed by binding to OVCA 433 cells before incubation with fluorescein-conjugated goat antimouse immunoglobulin. Fluorescence intensity of individual cells was measured on the FACS-1. After precipitation of immunoglobulin with zirconyl phosphate gel, the supernatant activity was reduced to background.

DISCUSSION

A monoclonal antibody has been developed that reacts with tumor cells from a majority of patients with ovarian carcinoma. Using indirect immunofluorescence, it has been possible to study the reactivity of this antibody with a number of nonmalignant tissues and with tumor cell lines established from a variety of neoplasms. To date OC125 has failed to bind to any of the nonmalignant adult or fetal tissues tested. Within the limitations of indirect immunofluorescence, the reactivity of OC125 appears restricted to malignant cells and to cell lines derived from them. OC125 binds to 7 of 11 specimens of cryopreserved ovarian ascites tumor, to 5 of 9 cryopreserved ovarian carcinoma sections, and 6 of 6 epithelial ovarian carcinoma cell lines, but to only 1 of 14 cell lines derived from nonovarian tumors. The only nonovarian tumor cell line to which OC125 has bound was derived from a malignant melanoma. OC125 failed to react with three other cell lines derived from melanomas, suggesting that the antibody is not directed against a determinant shared by a majority of malignant melanomas and ovarian carcinomas.

TABLE IV
Reactivity of OC125 and Other Monoclonal Reagents with Hematopoietic and Lymphoreticular Cells

Cell type	Monoclonal reagents			
	T3	B1	Ia	OC125
B (n = 5)	4±2*	86±3	88±3	2±1
T (n = 5)	87±1	2±1	3±1	2±1
Mono (n = 5)	5±2	3±1	56±8	4±2
Null (n = 5)	3±3	1±1	2±1	2±2
Tonsil (n = 3)	8±2	52±5	48±3	2±1
LN (n = 3)	46±5	37±7	45±6	1±1
BM (n = 3)	2±3	3±2	7±3	1±2

* Percentage of cells reactive±SD.

The relationship of the antigen recognized by OC125 to other ovarian tumor-associated antigens defined by conventional heteroantisera remains to be determined. Although the identity of the moieties with which OC125 reacts must await the outcome of biochemical analysis, the antibody did not coprecipitate radiolabeled CEA. This is an important control in that

circulating CEA can be detected in the peripheral blood of at least 40% of ovarian cancer patients during the course of their illness (37). Although elevated CEA is most frequently associated with tumors of mucinous histology, it can be associated with ovarian carcinomas that exhibit other morphologies.

Monoclonal antibodies are now being developed against antigens associated with several different human malignancies (30, 38–40). Similar reagents that react specifically with human ovarian carcinoma may prove particularly useful both for diagnosis and treatment of this disease. To date prognosis in ovarian carcinoma has been correlated with the degree of differentiation, clinical stage, and histological type of the neoplasm (41). Among serous cystadenocarcinomas of similar grade, OC125 has bound to some tumor specimens, but not to others. The presence or absence of antigens may permit more precise classification and prognostication in the ovarian carcinomas, as has been the case in studies of acute leukemia (42) and of lymphoma (43).

Cytological analysis of ascites specimens is a continuing problem for the pathologist. Antibodies that identify malignant tumor cells of ovarian origin in pleural or peritoneal fluid may prove valuable in surgical staging and in determining the probable site of primary malignancy. Considering the difficulties encountered in evaluating response to therapy, several investigators have attempted to develop immunoassays for the detection of tumor-associated antigen in peripheral blood or body fluids of patients with known ovarian carcinoma. The availability of large amounts of essentially pure antibody should facilitate development of such assays, provided that an antibody can be isolated of appropriate affinity as well as specificity for an antigen that is shed from the tumor cell surface. Antigens that are not shed may still prove useful as targets for *in situ* diagnosis using radiolabeled antibodies and for therapy using monoclonal antibodies that have been conjugated with cytotoxic agents or with isotopes at high specific activity.

Recent work from our laboratory suggests that treatment with a combination of specific heteroantiserum and immunostimulant is more effective than treatment with either single agent for eliminating ovarian tumor cells in a murine model for serotherapy (11). Synergy between antibody and *Corynebacterium parvum* appears to depend, at least in part, on the ability of the immunostimulant to attract, arrest, and activate peritoneal cells that mediate antibody-dependent cytotoxicity. With the availability of monoclonal reagents, it should be possible to evaluate a similar approach in clinical trials of intraperitoneal immunotherapy using antibodies of appropriate specificity and subclass in combination with immunostimulants.

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