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# Use of Selected Combinations of Monoclonal Antibodies to Tumor Associated Antigens in the Diagnosis of Neoplastic Effusions of Unknown Origin

M. MOTTOLESE, I. VENTURO, R. PERRONE DONNORSO, C. GALLO CURCIO, M. RINALDI and P.G. NATALI

*Istituto Nazionale Tumori 'Regina Elena', Viale Regina Elena* 291, 00161 *Roma, Italy*

**Abstract**—*While conventional cytodiagnosis can, in most instances, recognize cancer cells in metastatic effusions from solid tumors, the cellular type or the organ of origin of the primary neoplasia can rarely be determined only on the basis of their morphology. In the present study we have evaluated whether immunocytochemical techniques can be used to overcome this limitation by employing a panel of monoclonal antibodies (MoAbs) to tumor associated antigens (TAA) which lack detectable reactivity with mesothelial cells. To this end we have analyzed, by indirect immunofluorescence, cytospins of* 60 *malignant effusions of unknown origin. The results of this study have shown that the definition of the origin of the primary tumor, which was subsequently confirmed histologically and/or clinically, could be reached in* 87% *of the cases. These findings demonstrate that selected combinations of MoAbs, when used in immunocytochemical tests, can provide a powerful diagnostic tool in defining the site of cryptic primary neoplasias causing metastatic effusions.*

## INTRODUCTION

THE CYTOPATHOLOGIC diagnosis of metastatic effusions from solid neoplasms is often hampered by the presence of 'activated' mesothelial cells or macrophages, which can mimic tumor cells or conceal their presence [1], and by the cancer cell morphology, which may undergo substantial modifications from that observable in primary lesions [2]. While, in spite of these limitations, malignant cells can be detected in most instances by morphological criteria, the identification of the organ of origin of the primary tumor remains elusive as often emphasized by current literature in absence of a clear clinical picture [3–5]. Efforts to improve this diagnosis have been attempted by complementing conventional cytology with histochemical [6, 7], ultrastructural [8, 9] and chromosomal studies [10, 11]. More recently, the development of monoclonal antibodies (MoAbs) to tumor associated antigens (TAA) has provided an additional diagnostic tool [12–15] in this context. In the present study we have used a panel of MoAbs to TAA for the definition of the tumor origin of neoplastic effusions which had appeared in patients with no initial clinical evidence of malignancy. Data to be presented demonstrate that selected combinations of MoAbs when used in immunocytochemical tests are capable of identifying the primary tumor site in 87% of the instances.

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Address reprint requests to: Dr. P.G. Natali, Istituto Nazionale Tumori 'Regina Elena', Viale Regina Elena 291, 00161 Roma, Italy.
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## MATERIALS AND METHODS

*Patients*

Sixty-five patients with various malignancies evaluated in this study were admitted to the Regina Elena Cancer Institute in Rome because of the appearance of either pleural or peritoneal effusions during the clinical course of a previously diagnosed malignancy. Sixty additional cases which were of major interest in this study were referred with a diagnosis of neoplastic effusion of 'unknown origin'. While the first group was free of treatment for various lengths of time (3–6 months) the second category of patients was completely untreated. Flu-

ids, taken from 23 patients with proven non-malignant diseases, consisted of four peritoneal fluids and eight peritoneal washings from ovarian cystoadenomas, four peritoneal washings and two peritoneal fluids from uterine leyomioma, one peritoneal effusion from liver cirrhosis, four pleural effusions from pulmonary diseases. In addition we tested four pleural effusions from patients with lymphomas.

*Sampling of effusions and preparation of cell substrates*

Pleural and peritoneal effusions were collected in sterile conditions using heparin (Liquemin, Roche) as anticoagulant. Cells were harvested from the fluid by centrifugation at 160 ***g*** for 10 min. Cell pellets were washed three times with Hanks balanced salt solution (HBSS) (Gibco Lab., Paisley, U.K.) and resuspended in the same medium at a density of $1 \times 10^6$ cells/ml. When the effusions were highly contaminated by red blood cells, erythrocytes were removed prior to cell washing by lysis with Tris–$NH_4Cl$ pH 7.4 for 10 min at 37°C.

Cytospins were obtained using a Shandon cytocentrifuge (Shandon, Runcorn, Cheshire, U.K.) and used for conventional morphological diagnosis after staining with Papanicolou and May–Grünwald methods or fixed for 10 min in absolute acetone for immunocytochemical evaluation. After fixation the cytospins were either immediately processed or stored at −20°C for at least 4 months with no loss of serological reactivity.

*Monoclonal and polyclonal antisera*

MoAbs to various TAA were kindly provided by various investigators (MBRI, OC125, MOv19, KS1/4) or obtained commercially (B72.3, B6.2, Sorin Biomedica, Saluggia, VC, Italy) ([16–21], see Table 1). They were employed as purified antibodies [22]. A fluorescein labeled $F(ab)_2$ fraction of a goat anti-mouse immunoglobulin antiserum was obtained from Sorin Biomedica. Prior to its use on cytospins the antiserum was extensively absorbed with human ABRh+ red blood cells and with insolubilized pooled normal human plasma [23]. The antiserum was employed at a protein concentration of 500 μg/ml with a fluorescein–protein ratio of 3.

*Indirect immunofluorescence*

Indirect immunofluorescence (IIF) on acetone fixed cells was performed as follows: cytospins were incubated overnight with MoAbs to various TAA at 4°C. The protein concentration of primary antibody ranged from 25 to 50 μg/ml in HBSS containing 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, U.S.A.). After three washes with phosphate buffered saline (0.15 M) (PBS) cytospins were incubated for 30 min at room temperature with fluorescein labelled $F(ab)_2$ goat anti-mouse immunoglobulins antiserum. Following three washes with PBS cytospins were mounted with 50% buffered glycerol pH 7.2 and examined under a Leitz Orthoplan microscope equipped with epiillumination and phase contrast observation. Control cytospins were prepared by substituting the incubation with primary antibody with HBSS 1% BSA. Effusions were considered positive when at least 10% of the cell population analyzed was found immunocytochemically reactive in two separate cytospins or when less than 10% of the cells were found positive but the finding was consistent in cytospins obtained from separate taps.

The immunocytochemical findings were evaluated independently by two investigators with no knowledge of the cytopathological diagnosis. The serological findings were compared with those of the histopathologic examination when available and/or with the clinical data obtained following patients' clinical diagnostic procedures.

## RESULTS

*Monoclonal antibodies*

MoAbs used in this study which are shown in Table 1 were chosen on the basis of two main serological properties. They recognize TAAs which are not coordinately expressed by transformed cells and lack detectable reactivity with mesothelial cells.

MoAb B72.3 defines a high molecular weight glycoprotein highly associated with the malignant phenotype and selectively expressed by most adenocarcinomas [16, 17]. The other reagents, although not possessing a strict tumor specificity, recognize TAAs mainly expressed by breast (B6.2 85%,

*Table 1. Monoclonal antibodies used for the immunocytochemical analysis of serous effusions*

| Antibody | Isotype | Antigen | Molecular weight | Major reactive tumor | References |
|---|---|---|---|---|---|
| B72.3 | IgG1 | Glycoprotein | $>10^6$ d | Adenocarcinomas | [16, 17] |
| B6.2 | IgG1 | Glycoprotein | $9 \times 10^6$ d | Breast carcinoma | [16] |
| MBR1 | IgM | Glycolipid | | Breast carcinoma | [18] |
| OC-125 | IgG1 | Glycoprotein | $0.5 \times 10^6$ d | Ovarian carcinoma | [19] |
| MOv19 | IgG2a | Glycoprotein | $3.8 \times 10^6$ d | Ovarian carcinoma | [20] |
| KS1/4 | IgG2a | Glycoprotein | $4 \times 10^6$ d | Lung carcinoma | [21] |

MBR1 (80%) [16–18], ovarian (OC-125 80%, MOv19 80%) [19, 20] and lung (KS1/4 95%) carcinomas [21]. Among these reagents only MoAb MOv19 displays the most restricted type of tumor expression since the TAA recognized by this reagent is shared only by a minority (2–10%) of breast carcinomas.

*Reactivity of a panel of MoAbs to TAA with neoplastic effusions of known histotype. Definition of diagnostic patterns of reactivity*

The first part of the present study was performed with the aim of verifying whether a panel of MoAbs to TAA expressed by tumors frequently producing metastatic effusions could be of diagnostic value when employed in immunocytochemical tests. This initial study was performed on 65 pleural and peritoneal effusions sampled from patients with an established tumor diagnosis and free of treatment for at least 3 months. The immunocytochemical findings of this analysis are summarized in Table 2. The lack of reactivity of the majority of the MoAbs employed in this study with mesothelial cells and macrophages was supported by the current literature [13, 14]. We nevertheless performed control stainings of non-neoplastic effusions and peritoneal washings from patients with non-malignant diseases and non-epithelial tumors. None of the control specimens was found to be reactive with the panel of MoAbs used except for MoAb B6.2 which, as expected, identified polymorpholeukocytes (Table 3). In this context it may be noted that we did not observe any staining of benign mesothelial cells by OC-125 as reported by Kabawat *et al.* [24] in their immunohistochemical study of pleural and peritoneal biopsies. In most instances tumor cells within one metastatic effusion displayed various degrees of reactivity with MoAbs ranging from negative to strongly positive (Fig. 1A–D). All these effusions tested reacted with MoAb B72.3. The other reagents, when considered individually, displayed a preferential reactivity with a tumor type but none of them except for MoAb MOv 19 appeared strictly 'tumor specific', on these cell sub-

*Table 2. Immunocytochemical reactivity of pleural and peritoneal effusions from patients with known primary tumor with a panel of monoclonal antibodies to tumor associated antigens*

| Primary tumor diagnosis | Number of patients | Patterns of reactivity with MoAbs | | | | | |
|---|---|---|---|---|---|---|---|
| | | B72.3 | B6.2 | MBR1 | MOv19 | OV-125 | KS1/4 |
| Breast carcinoma | 15 | + | + | + | – | – | – |
| | 3 | + | + | – | – | – | nt* |
| | 5 | + | – | + | – | – | + |
| | 2 | – | + | + | – | + | – |
| | | 23/25† | 20/25 | 22/25 | 0/25 | 2/25 | 5/22 |
| Ovarian carcinoma | 6 | + | – | – | + | – | + |
| | 3 | + | + | + | + | + | + |
| | 11 | + | – | – | + | + | – |
| | 4 | + | – | + | + | + | – |
| | 6 | – | – | – | + | + | + |
| | | 24/30 | 3/30 | 7/30 | 30/30 | 24/30 | 15/30 |
| Lung carcinoma | 2 | – | – | + | – | – | + |
| | 8 | + | – | – | – | + | + |
| | | 8/10 | 0/10 | 2/10 | 0/10 | 8/10 | 10/10 |

*nt: not tested.
†No. positive/No. tested.

*Table 3. Immunodiagnostic patterns of reactivity of neoplastic effusions with monoclonal antibodies to tumor associated antigens*

| Immunocytochemical diagnosis | Patterns of reactivity with MoAbs | | | | | |
|---|---|---|---|---|---|---|
| | B72.3 | B6.2 | MBR1 | MOv19 | OC-125 | KS1/4 |
| Metastatic breast carcinoma | + | + | + | – | – | ns* |
| Metastatic ovarian carcinoma | + | – | – | + | + | ns |
| Metastatic lung carcinoma | + | – | – | – | + | + |
| Non-malignant effusions† | – | +‡ | – | – | – | – |

*ns: not significant
†See Materials and Methods.
‡Only on polymorpholeukocytes.

strates. No significant differences in immunocytochemical reactivity were seen between pleural and peritoneal effusions.

The data of Table 2 demonstrate also that when the reactivity of a given specimen with all the reagents tested was taken into account, distinct patterns of reactivity could be identified. Each serological pattern when matched with the patient's known clinical pathological data consistently identified tumors of different origin.

These diagnostic patterns of reactivity are summarized in Table 3. Because of its consistent reactivity, MoAb B72.3 was a useful general marker of malignancy to detect neoplastic cells thus contributing to substantiate the positive reactivity of other MoAbs. In fact, only two cases of ovarian carcinoma were found unreactive with this reagent. Metastatic breast and ovarian carcinoma were identified by the patterns B6.2$^+$, MBRI$^+$, MOv 19$^-$, OC 125$^-$ and B6.2$^-$, MBRI$^-$, MOv 19$^+$, OC-125$^+$, respectively. The reactivity of cells with the MoAb KS1/4 in conjunction with MoAb OC-125, which is known to react also with non-small cell lung cancers [25], was highly suggestive of a metastatic lung tumor, especially in male patients where ovarian tumors could be excluded.

*Immunocytochemical definition of the tumor origin of neoplastic effusion from patients with cryptic primary tumor*

Once the serological patterns of reactivity of Table 3 were defined, we submitted 60 cases of pleural and peritoneal effusions present in patients with no initial clinical evidence of a primary tumor to the same immunocytochemical analysis. The immunocytochemical diagnosis reached was compared with conventional cytologic findings and clinical–pathological data which were collected upon further evaluation of the patients. The results of this study are reported in Table 4. Of the 60 patients studied, an immunocytochemical diagnosis suggesting the organ of origin of the metastatic effusion, was reached in 56 (87%) of the instances. In all these patients the clinical follow-up confirmed the site of the primary tumor except for three cases of lung and one case of ovarian tumor which on the basis of their serological reactivity were indicated as breast and lung tumor respectively. In four patients the immunocytochemical diagnosis could not be detailed beyond that of 'metastatic carcinoma'. They were found to bear an ovarian, a mammary and two pulmonary carcinomas.

## CASE REPORTS

Two clinical cases in which the immunocytochemical findings employing our panel of MoAbs have been helpful in reaching a conclusive diagnosis are reported. In both instances the effusions were referred to as 'of unknown origin'.

*Case* 1

Patient M.L. was a 50 year old female who presented a peritoneal effusion and a completely negative clinical examination. Cytological studies of the fluid revealed 'atypical cells'. When submitted to immunocytochemical analysis with the panel of MoAbs the cytospins gave the following pattern of

*Table* 4. *Immunocytochemical definition of the tumor type of neoplastic effusions from patients bearing unknown primary tumor*

| Number of patients | Patterns of reactivity with MoAbs B72.3 | B6.2 | MBR1 | MOv19 | OC-125 | KS1/4 | Immunocytochemical diagnosis | Histologic and/or clinical diagnosis |
|---|---|---|---|---|---|---|---|---|
| 10 | + | + | + | − | − | − | Metastatic breast carcinoma | Metastatic breast carcinoma |
| 3 | + | − | + | − | − | − | Metastatic breast carcinoma | Metastatic breast carcinoma |
| 2 | + | + | + | − | + | + | Metastatic breast carcinoma | Metastatic breast carcinoma |
| 1 | + | − | + | − | − | + | Metastatic breast carcinoma | Metastatic breast carcinoma |
| | | | | | | | | 13/16 confirmed |
| 10 | + | − | + | + | + | + | Metastatic ovarian carcinoma | Metastatic ovarian carcinoma |
| 2 | − | − | + | + | + | + | Metastatic ovarian carcinoma | Metastatic ovarian carcinoma |
| 2 | + | + | − | + | − | − | Metastatic ovarian carcinoma | Metastatic ovarian carcinoma |
| 5 | + | − | − | + | + | − | Metastatic ovarian carcinoma | Metastatic ovarian carcinoma |
| 5 | + | − | − | + | + | − | Metastatic ovarian carcinoma | Metastatic ovarian carcinoma |
| | | | | | | | | 24/24 confirmed |
| 8 | + | − | − | − | + | + | Metastatic lung carcinoma | Metastatic lung carcinoma |
| 2 | + | − | − | − | − | + | Metastatic lung carcinoma | Metastatic lung carcinoma |
| 5 | − | − | − | − | + | + | Metastatic lung carcinoma | Metastatic lung carcinoma |
| 1 | + | − | − | − | − | + | Metastatic lung carcinoma | Metastatic lung carcinoma |
| | | | | | | | | 15/16 confirmed |
| 2 | + | + | + | − | + | + | Undefined carcinoma | Metastatic carcinoma |
| 2 | + | − | − | − | − | − | Undefined carcinoma | Metastatic ovarian, metastatic breast carcinoma |

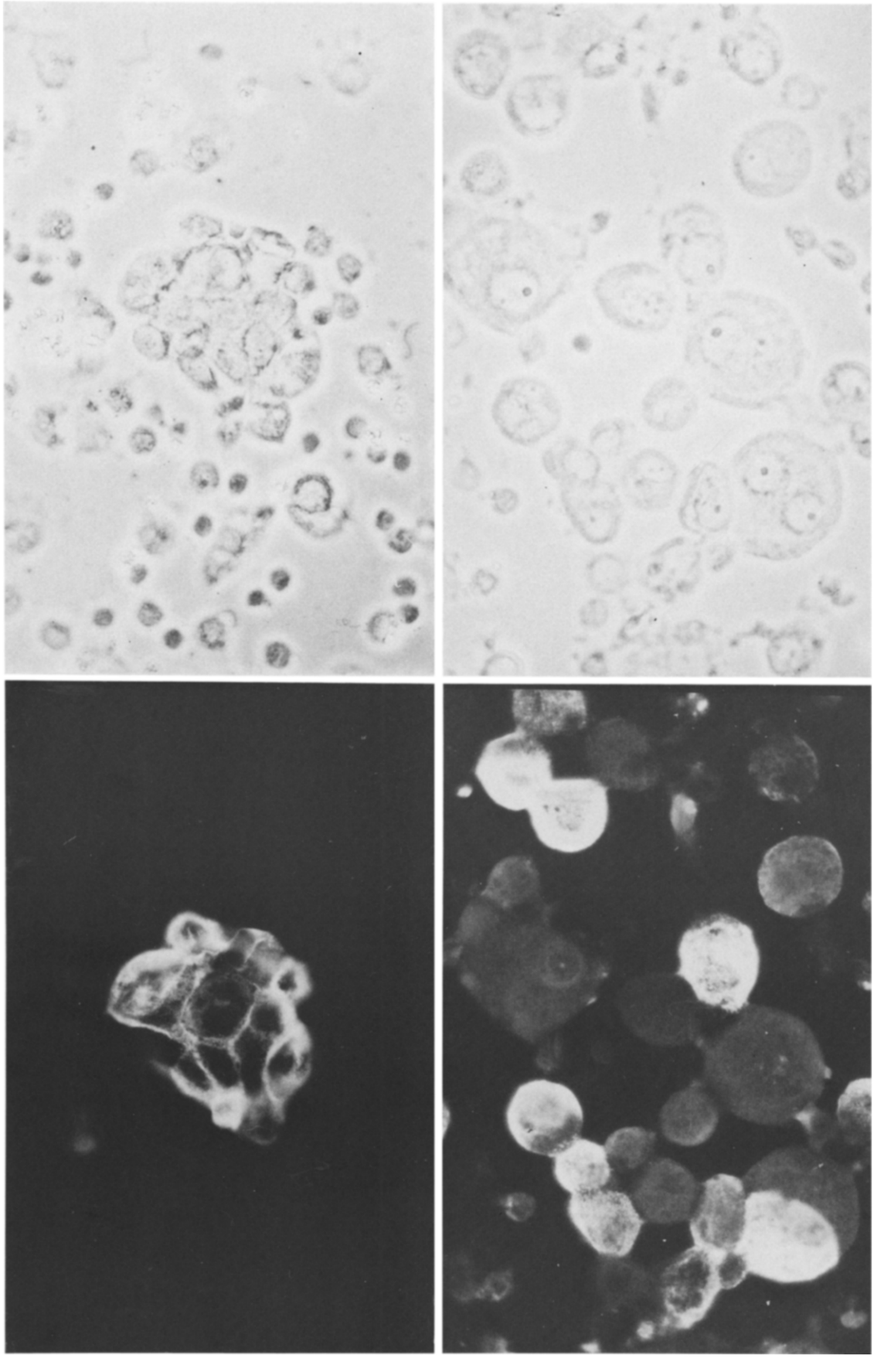

***Fig.* 1. *Acetone fixed cytospins analyzed by indirect immunofluorescence and phase contrast microscopy of a pleural (a,c) effusion from breast carcinoma and a peritoneal (b,d) effusion from an ovarian carcinoma. The specimens display a homogeneous and heterogeneous reactivity with MoAb B72.3 and with MoAb MOv19 respectively (× 520).***

reactivity: B72.3+, B6.2+, MBR-1+, MOv 19−, OC-125−, which suggested a metastatic breast carcinoma. The mammography which was then performed showed a suspect area of 15 mm in the left breast which upon tumorectomy was diagnosed as 'infiltrating ductal carcinoma'.

*Case* 2

Patient G.L. was a 51 year old female with no past history of malignancy who presented a pleural and a peritoneal effusion which, on a CAT scan, was found to be associated with a normal pelvis. Cytology of the fluid revealed 'metastatic carcinoma'. The immunocytochemistry displayed the following reactivity: B72.3+, B6.2−, MBR-1−, MOv 19++, OC-125+, suggesting a metastatic ovarian carcinoma. The patient was then submitted to an abdominal echography which revealed a solid mass of 35 mm in diameter on the right adnexa and to a determination of OC-125 serum levels which were found to be 88.5 U/ml. At a staging laparotomy a papillary cystoadenocarcinoma of the ovary was found.

## DISCUSSION

The presence of a pleural and/or peritoneal effusion in a patient free from an infectious disease and a hematologic malignancy raises two main questions to the cytoscreener. Are neoplastic cells present? If so, where do they originate from? While a number of efforts to apply immunocytochemical tests, in this context using MoAbs, have been found to improve the detection of malignant cells in tumor recurrences [12–15, 26–28], little has been done to evaluate whether the same approach can identify the origin of metastatic cells in patients with no previous malignancy. In the present study we have shown that the cytopathologic diagnosis of metastatic effusions from solid tumors can be strikingly improved in establishing the site of the primary tumor. This can be achieved by using appropriate combinations of MoAbs which recognize TAA and lack detectable reactivity with mesothelial cells in immunocytochemical tests which are easy to perform and can be objectively interpreted. The use of more than one reagent is required for two main reasons. Firstly, most of the MoAbs to TAA do not possess a strict tumor type specificity and secondly the majority of the TAA recognized by the reagents have a heterogeneous expression within a tumor cell population. Despite these limitations, a relatively small number of reagents is capable of reaching a conclusive serologic diagnosis (87%) of the most commonly occurring neoplastic effusions from solid tumors. A larger number of cases which will include other malignancies will be needed to establish the extent of application of this method. At the present stage of development some limitations of the test should be commented on. Among the pleural effusions studied, those originating from lung tumors because of the unavailability to us of highly specific MoAbs could be firmly diagnosed predominantly in male patients. In fact, in females the cross-reactivity of MoAb KS1/4 and OC 125 with breast and ovarian tumors respectively provides questionable although still diagnostically informative findings. The addition to the panel of MoAbs used in this study of other already available or new MoAbs is expected to expand and to improve the application and accuracy of this diagnostic approach. The present method appears to offer a valuable guide to the physician in choosing more targeted diagnostic examinations during the clinical evaluation, thus saving time and patient distress. Furthermore, the method can be of value, as it is already in our experience, in selecting appropriate therapies for those patients who become affected by a second malignancy. We are currently evaluating whether the serologic reactivity of tumor cells with our panel of MoAbs is undergoing changes following therapy. If this is not the case, this method could also be applied to establish the presence of residual disease or the occurence of relapses.

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