CITOLOGÍA DEL MESOTELIOMA

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El mesotelioma presenta un amplio rango de caracteres citomorfológicos y una amplia variedad histológica de patrones de crecimiento, divididos en las categorías epitelioide, el más común entre 60 y el 80%, sarcomatoide supone entre el 15 y el 20% o mixto alrededor del 30%, cada una de estas categorías se puede subclasificar.

Aproximadamente el 50% de los mesoteliomas pleurales y el 75% de los peritoneales son de tipo epitelial, mientras que el 25% y el 15% respectivamente son de los tipos bifásicos o sarcomatoides, los casos restantes son pobremente diferenciados o subtipos especiales. Este último apartado incluye una gran variedad de mesoteliomas lo que conlleva diferentes diagnósticos diferenciales, son poco comunes y generalmente no estamos familiarizados con su apariencia citológica, sin embargo es importante conocer su existencia y levantar la sospecha de una variante infrecuente en casos problemáticos.

El proceso del diagnóstico citológico de mesotelioma epitelial, que presenta dificultades principalmente debido a la significativa superposición de aspectos citológicos entre las células mesoteliales benignas y malignas, y entre el mesotelioma y las células del adenocarcinoma, conlleva dos pasos. En el primero hay que reconocer las células como de linaje mesotelial. En el segundo hay que reconocerlas como neoplásicas. Generalmente no es difícil percibir su origen mesotelial en las células pero aunque están descritos criterios citológicos para el diagnóstico puede ser difícil de decidir si son malignas porque los caracteres de malignidad pueden estar pobremente representados. El reconocimiento de otra celularidad, no inflamatoria, distinta a las células mesoteliales es el carácter citológico más importante para hacer el diagnóstico de metástasis de carcinoma y es en esta situación cuando la inmunocitoquímica es de más ayuda.

Otras dificultades están en la diferenciación de las muestras tomadas por PAAF, entre el mesotelioma sarcomatoide y otros tumores de células fusiformes y entre el mesotelioma mixto y el sarcoma sinovial.

Ningún marcador tiene el 100% para la sensibilidad y especificad del mesotelioma, por lo que en el enfoque de la inmunocitoquimica, es adecuado usar un panel de anticuerpos positivos y negativos que varía dependiendo del tipo histológico del mesotelioma (epitelioide versus sarcomatoide), la localización del tumor (pleura o peritoneo) y el tipo de tumor que es considerado en el diagnóstico diferencial. Estos paneles están cambiando continuamente como resultado del reconocimiento de nuevos marcadores. Inmunocitológicamente el EMA, y la p53 se expresan en el mesotelioma mientras que la desmina es más común en el mesotelio reactivo.

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LIQUIDO ASCITICO Y USO DE INMUNOCITOQUIMICA

ASCITIC FLUID: USE OF IMMUNOCYTOCHEMISTRY

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The major purpose of cytologic examination of serous effusions is to determine whether malignant cells are present. This is an extremely important task since in most cases the presence of malignant cells in ascitic or pleural effusions indicates an advanced or terminal stage of malignancy. Cytologic diagnosis may be difficult and many diagnostic pitfalls exist. False positive results may prevent a necessary surgery and false negative results lead to unnecessary treatment.

In order to avoid diagnostic pitfalls, special attention should be given to proper collection, preparation and fixation of cytologic material. Diagnostic criteria should be followed carefully and, in equivocal cases, when the diagnostic material is inadequate or poorly preserved, repeat cell study should be recommended. Knowledge of the complete clinical history, patient's age, sex, and occupation, prior history of cancer and treatment modalities such as radiation and/or chemotherapy should be available for proper interpretation and meaningful clinicopathological correlation. In this review, we will first discuss a simplistic approach in the diagnosis of serous effusions (pleural, pericardial and peritoneal) with emphasis on the cytomorphologic criteria found in alcohol-fixed Papanicolaou-stained cytospin slides. The cytomorphologic diagnosis of cerebrospinal fluid specimens (CSF) will follow our general discussion on the serous fluid specimens. In this handout we have also provided cytopreparatory techniques and the modified Papanicolaou staining method used in our laboratory (See supplements I and II). Please note that when needed, we use immunocytochemistry on our previously Pap-stained slides, without destaining (See supplement III).

Serous Fluid; Its Source and Cellular Content

The presence of malignant cells in serous effusions indicates involvement of serous membranes by cancerous cells. However, many other conditions cause the accumulation of fluid in the body cavities, such as inflammation, congestive heart failure, cirrhosis of the liver, renal failure, trauma and the presence of any long standing, space occupying mass (usually benign) within the serosal cavities.

In the absence of cancerous implants, effusions are mainly caused by the obstruction of vascular and lymphatic channels, impairing the reabsorption of fluid from the cavity. Due to the chronic irritation of serous surfaces, a varying degree of mesothelial proliferation occurs. Exfoliated mesothelial cells often demonstrate changes that may be confused for malignancy. These benign mesothelial cells will be referred to as "reactive" mesothelial cells.

Mesothelial cells: Mesothelial cells are present in the majority of effusions, but especially in sterile inflammations. They are absent or scarce when a serous cavity becomes infected by pyogenic organisms. Mesothelial cells that shed into the serous cavities appear in clusters or isolated. When these cells are aspirated during a surgical procedure (as in peritoneal washings), they are more commonly found in sheets, presumably as result of their forceful detachment from the serosal surfaces. Mesothelial cells exfoliated from the pericardium, on the other hand, have a tendency to roll up due to the constant cardiac movements. This may present a diagnostic pitfall when dealing with pericardial fluid specimens of patients with benign accumulation of pericardial fluid. Reactive mesothelial cells are arranged in loose groups, and tight molding cellular clusters are not usually present. The cytoplasmic borders are sharp and usually remain intact. In sheet-like arrangements, the cells have a mosaic pattern. Isolated mesothelial cells may also be present in variable number. They are round or oval measuring 10-12 microns. The cytoplasm is dense and two-toned. A foamy rim may be present in some cells; sometimes brush borders are conspicuous. The nuclei are usually centrally located. Binucleation or multinucleation is not uncommon. In binucleated cells, the nuclei are mirror images of each other. The nuclei are round or oval with a smooth nuclear membrane, and evenly distributed chromatin. Nucleoli may be prominent. Degenerated mesothelial cells contain cytoplasmic vacuoles resembling macrophages.

Macrophages: Macrophages are found in almost every serous fluid. They are usually larger than mesothelial cells and contain a bean-shaped nucleus. The cytoplasm is foamy and may contain phagocytized material or large vacuoles displacing the nucleus to the periphery "signet ring cell". Degenerating mesothelial cells may be confused with macrophages, however, their cytomorphologic distinction is not of any clinical significance.

Red Blood Cells: A bloody aspirate is rare in non-traumatic fluid collections except in neoplasia.

Eosinophils: The most common cause of eosinophils in serous fluid is repeated taps and subsequent exposure of serous membranes to the air. Eosinophils are also seen in allergic conditions and are rarely associated with neoplasms.

Lymphocytes: The presence of numerous mature lymphocytes in serous fluid usually represents tuberculosis, in which usually more than 50% of the specimen is composed of lymphocytes. Lymphocytes are also seen in non-specific inflammatory processes.

Other cells: In addition to mesothelial cells, other benign epithelial cells may be found in

serous effusions, most commonly, in peritoneal wash specimens. Columnar or cuboidal cells of Mullerian origin may be found in cases of endosalpingiosis. Presence of Psammoma bodies alone is not indicative of a serous neoplasm. Rarely, epithelial cells from organs located at the site of aspiration (such as liver) may be found in aspirated fluids.

Is this a malignant effusion?

The diagnosis of malignancy in effusions should be attempted based on cytomorphology and should be followed by additional studies, if needed. We try to avoid the diagnosis of "atypical cells" in our daily practice. The "atypical" diagnosis is meaningless for the clinician who is dealing with a patient with known or unknown primary malignancy that has presented with a serous effusion. We try to be conclusive in our cytologic diagnosis by performing ancillary techniques such as immunocytochemistry and flow cytometry.

Based on cytomorphologic findings alone, the first and the most important question to answer is if the effusion represents a malignant process. An extremely simplistic approach to the diagnosis of a fluid specimen is to observe the following:

A. Cellular pattern (cells in cluster or isolated)

B. Cell morphology

A. Cellular Pattern

1. **When the majority of cells are in clusters**: In these cases, the malignant cells can easily be detected, even at the low power magnification. The clusters of malignant cells are tight and compact, usually with smooth borders. The cells mold, each taking the shape of the neighboring cell. In the majority of these cases, low power evaluation will show two distinct cell types: malignant cells within the tight clusters and reactive mesothelial cells (isolated, in pair or in small sheets) in the smear background. Reactive mesothelial cells may also group, however, the grouping is usually loose and nuclei do not overlap. In such reactive conditions, nuclear features of the cells in clusters are identical to those of isolated or paired mesothelial cells seen in the smear background.
2. **When the majority of malignant cells are isolated:** Poorly differentiated adenocarcinomas in effusion specimens are the most difficult to diagnose. Unless the isolated cells are obviously abnormal and show cytomorphologic characteristics of malignancy (pleomorphism, high nucleocytoplasmic ratio, hyperchromasia, abnormal nucleoli and clumped, irregular chromatin), these tumor cells may be absolutely overlooked on low power evaluation. Typical examples of this group are metastatic breast carcinomas and poorly differentiated adenocarcinomas from the gastrointestinal tract (GI). In such cases, careful search for *tightly grouped cell clusters* not only will confirm the diagnosis of malignancy, but also will indicate the epithelial origin of the primary tumor. Less commonly encountered are malignant effusions that are known to produce isolated cells in cytologic material (usually in aspiration cytology). These

include malignant melanomas, lymphomas and sarcomas, their specific cytomorphologic characteristics will be discussed later.

C. **Cellular Morphology:** There are cases in which the diagnosis of malignancy can easily be made based on the presence of cells, demonstrating established cytologic criteria regardless of their cellular arrangement. In contrast, the diagnostic problem is profound when no tight clusters are found and the cells are only seen in isolated forms without convincing cytomorphologic characteristics of malignancy. This is especially common in cases of metastatic lobular carcinoma of breast, when tumor cells are small with bland nuclei, and lack striking features of malignancy. In such cases the use of immunocytochemistry is often very helpful for a definitive diagnosis. A diffuse intracytoplasmic staining for epithelial membrane antigen (EMA) is extremely helpful in such instances. In addition, immunostaining for estrogen receptor (ER) usually confirms the breast origin in such cases.

***Useful* Findings:** tight clusters with smooth borders; cellular and nuclear molding; large papillary groups; two- cell types; "signet ring "cells in groups; nuclear abnormalities.

***Useless* Findings:** cytoplasmic vacuoles; "signet ring" cells; cell within cell; prominent nucleoli; psammoma bodies; mitosis; multinucleation.

<u>It is malignant; what type?</u>

When the diagnosis of malignancy is established (based on cellular pattern and cell morphology), the next important diagnostic step is to narrow down the diagnosis to the cell type and further investigate the possible site of origin. This should be attempted by complete knowledge of the patients' clinical history.

The most common type of malignancy encountered in serous effusions is adenocarcinoma. In ascitic fluid of adult male, GI and lymphoma are the most frequently encountered neoplasms. In adult female, ovary is the most frequent site of origin, followed by breast, GI and lymphoma. Malignant Cells in Effusions;

Adenocarcinoma: In the majority of cases, establishing the diagnosis of adenocarcinoma is easy. The tumor cells are usually tightly clustered and easily recognized on low power magnification. Cell clusters in adenocarcinoma may have smooth or irregular borders. The cellular molding and nuclear overlapping are usually prominent. Large cytoplasmic vacuoles maybe seen; this finding can be due to cytoplasmic degeneration and does not necessarily indicate mucin production, thus we try to avoid using the term "mucinous adenocarcinoma". Interestingly the typical cases of mucinous adenocarcinomas seen in peritoneal cavity (pseudomyxoma peritonei) are rarely diagnosable cytologically; mainly due to the paucity of cytologic material and lack of cellular characteristics for malignancy in these well differentiated mucinous tumors. A few specific features are helpful in suggesting the origin of adenocarcinomas. The finding of papillary clusters

is most common in ovarian, lung, and rarely in thyroid carcinomas. Intracytoplasmic mucin is seen in many adenocarcinomas but especially in GI-tract, pancreas, mucinous ovarian, and lung primaries. Psammoma bodies are often seen in serous papillary tumors of female genital tract. "Signet Ring" Cells may be the result of cytoplasmic mucin pushing the nucleus against the cell membrane. The vacuoles may contain neutrophils. Only "signet ring "cells that show nuclear abnormalities and contain mucin are considered to be of carcinomatous origin (more commonly from breast, GI-tract, and ovary). Since degenerated mesothelial cells and/or macrophages may also appear as "signet ring" the diagnosis of mucin producing adenocarcinoma should not be made unless the "signet ring" cells are in groups and show nuclear abnormalities. When cells are arranged in a row ("indian filing"), the possibility of breast, GI-tract, and pancreatic adenocarcinomas should be considered. Although using the above morphologic characteristics are helpful in detection of the site of origin for the adenocarcinoma cells found in effusion specimens, today this diagnostic exercise is done with higher specificity utilizing commonly available immunocytochemical markers. Markers used for adenocarcinomas of colonic origin: CK20 (positive), CK7 (negative). Marker used for breast carcinomas: Estrogen receptors. Markers used for adenocarcinomas of lung origin: Thyroid Transcription Factor (TTF-1).

Squamous cell carcinoma: Squamous cell carcinomas are common primary tumors, mainly arising from lung, female genital tract, upper respiratory tract, skin and esophagus. However, finding squamous cell carcinoma cells in cavity fluids is an uncommon event. This may be due to the fact that squamous cell carcinomas do not shed cells frequently, or when they do, the cells are mistaken for other neoplasms such as poorly differentiated adenocarcinomas and malignant mesotheliomas. In keratinizing squamous cell carcinomas, the cells are pleomorphic, the cytoplasm contains keratin and nuclei are pyknotic or coarsely granular. Tadpole cells, keratin pearls and anucleated cells may be found. Non-keratinizing squamous cell carcinomas may be difficult to differentiate from other metastatic tumors on the basis of morphology alone. Presence of degenerative cytoplasmic vacuoles in the tumor cells. Patient's age, sex, and knowledge of previous cancer history and treatment are important for proper diagnosis. P63 is the marker used for squamous cell carcinomas.

Small cell carcinoma: Cytologic characteristics of small cell carcinomas are well recognized. The cells are small and may group in very tight cell balls in the fluid specimens. Nuclear molding is usually striking. The cellular pattern may simulate the" indian file "arrangement seen in breast cancer. The nuclei have coarse chromatin and wrinkled nuclear membrane. Occasional cells with nucleoli may be seen. The cytoplasm is very scant, therefore, when cells are not clustered, they are easily overlooked. Small cell carcinomas show a characteristic dot-like immunostaining with cytokeratin. These tumors should be differentiated from other small cell malignancies including malignant lymphoma (DC45, CD20, CD3 and other lymphoid markers), embryonal rhabdomyosarcoma (desmin and myogenin), neuroblastoma (synaptophysin and neuroblastoma antigen), Ewing's sarcoma / peripheral PNET (CD99) and mesenchymal chondrosarcoma (S100

protein and CD57). In the absence of a known primary tumor, the diagnosis of small cell malignant neoplasm can be followed by this series of immunocytochemical stains. Accurate cytologic subclassification of small cell malignant tumors relies heavily on knowledge of the patient's sex, previous history, and immunocytochemical findings.

Malignant lymphoma: Cytologic examination is an accurate method for the diagnosis of malignant lymphoma in effusions with an overall diagnostic accuracy of 45-88%. With increasing number of patients with AIDS, the diagnosis of malignant lymphoma in serous fluids has become a more demanding job for the cytopathologist. Malignant lymphomas are characterized by the presence of numerous isolated cells in the cytospin preparation of the serous fluids. The nuclei may show variation in size and shape. Indentation and convolution of the nuclei and the presence of prominent nucleoli are important diagnostic features of malignant lymphomas. Individual cell necrosis (apoptosis) is a peculiar and, in our experience, a very useful finding in cases of malignant lymphomas. When apoptotic cells are numerous on low power magnification, they can be confused with neutrophils. An erroneous diagnosis of acute inflammation should be avoided in such cases. Apoptosis may be the result of previous chemotherapy, however, in our experience it has also been found in the absence of treatment history. The cytoplasm of lymphoma cells is usually scant, basophilic and rarely well preserved. When serosal membranes are involved by diseases associated with proliferation of lymphocytes such as well differentiated small cell lymphocytic lymphoma, chronic lymphocytic leukemia (CLL), and Waldenström's macroglobulinemia, the effusion may contain a large number of mature looking lymphocytes. In such cases, an unequivocal separation of these neoplastic processes from chronic non-specific inflammation or tuberculosis is not possible on the basis of cytomorphology alone. Clonal proliferation of T or B lymphocytes can be determined by immunocytochemical stains for CD3 and CD20, or by flow cytometry. In our laboratory, we do not diagnose malignant lymphoma in serous effusions unless the cells have nuclear characteristics of malignancy and a large number of malignant cells are present. When the number of abnormal lymphoid cells is not considered to be sufficient for an unequivocal diagnosis of a malignant lymphoproliferative process, the following diagnosis is rendered; "Cellular evidence of atypical lymphoproliferative process; recommend further studies to rule out malignancy". In such instances a follow up specimen is sent for flow cytometry and/or for gene rearrangement studies. When a large number of mature lymphocytes are present in the presence of a clinical diagnosis of well differentiated small cell lymphoma or CLL, we report these findings as "numerous lymphocytes are present, consistent with..." Additional studies may follow for confirmation. Large cell and anaplastic lymphomas should be differentiated from other large cell malignant neoplasms such as anaplastic carcinomas of lung, pancreas and thyroid origin. In younger patients germ cell malignancies (seminomas/germinomas) should always be considered in the differential diagnosis. In such cases placental alkaline phosphatase (PLAP) may be used by immunocytochemistry.

In summary, the presence of isolated cells with irregular nuclear membrane, nuclear clefts, multiple irregular nucleoli and individual cell necrosis (apoptosis) should suggest the diagnosis of malignant lymphoma. Immunocytochemistry for LCA, keratin, S100, and PLAP will be helpful to differentiate lymphomas from undifferentiated carcinoma, malignant melanoma, and malignant germ cell tumors (seminomas/germinomas) respectively.

In Hodgkin's lymphoma, the cytologic picture is usually nonspecific and composed of a mixture of small mature lymphocytes and occasional larger mononuclear cells. Primary diagnosis of Hodgkin's lymphoma can only be made by finding abnormal mono or multinucleated Reed-Sternberg cells. The patient's previous history and use of immunocytochemical stains such as CD15 and CD30 may prove to be helpful in these cases.

Other tumors: Other malignant neoplasms may occasionally involve serous membranes, such as malignant melanoma and sarcomas.

Malignant melanoma presents predominantly as isolated cells or cells in small loose clusters. Binucleation and multinucleation are common. The nucleoli are large and may be multiple. Presence of intranuclear cytoplasmic inclusions and intracytoplasmic melanin are of diagnostic value. Diagnosis of amelanotic melanomas may be facilitated by using markers such as HMB45, S100 protein, melan-A, and tyrosinase.

Sarcomas are rarely found in serous effusions. Cytologic findings depend on the morphology of the primary tumor. High-grade spindle cell sarcomas present as isolated, pleomorphic spindle cells with irregular nuclear contour and large nucleoli. In low-grade spindle cell sarcomas, a definitive diagnosis of malignancy may not be possible due to the bland cellular morphology. Such cases are usually diagnosed as "spindle cell neoplasms, not further classified". Round cell sarcomas should be differentiated from undifferentiated carcinomas and melanomas. Knowledge of clinical history is extremely important in order to make the appropriate diagnosis of these uncommon tumors. Differential diagnosis of embryonal rhabdomyosarcoma, mesenchymal chondrosarcoma and Ewing's sarcoma/PNET with more commonly found small cell carcinomas in the body cavity fluid is mainly done based on the clinical history and immunocytochemical staining for desmin, myogenin/S100 protein/ CD99, and cytokeratin respectively.

Malignant mesothelioma

Malignant mesotheliomas are divided into epithelioid, sarcomatoid and mixed types. Benign mesotheliomas are fibrous and are classified as fibromas of serous membranes. They usually do not produce an effusion. In contrast, malignant mesotheliomas are usually epithelioid or mixed types and are commonly associated with effusions. The term "mesothelioma" will be used here to refer to the malignant epithelioid type. Microscopic diagnosis of malignant mesothelioma must be made in conjunction with radiologic / or gross

findings.

In body cavity fluid specimens, the cells of mesothelioma occur singly, in small or large three-dimensional clusters. They are cuboidal or polygonal, usually larger than reactive mesothelial cells and vary in size. The cells in clusters or small sheets may show intercellular spaces or windows, similar to what is seen in reactive mesothelial cells. "Cells within Cell" may be seen. The individual cell morphology resembles those of the reactive mesothelial cell including dense, two-tone cytoplasm, peripherally located vacuoles (blebs) and brush borders. When large cytoplasmic vacuoles are present, the nucleus is pushed to the periphery of the cell giving the appearance of a "signet ring" cell which should be differentiated from adenocarcinoma cells. Multinucleation is common. The nuclei are usually centrally located. Since the nuclei and amount of cytoplasm are both increased, N/C ratio is usually unchanged (This is in contrast to cases of adenocarcinomas). Nucleoli may be single or multiple and prominent. Intranuclear inclusions may be seen.

<u>Problems Associated with the Diagnosis of Malignant Mesothelioma</u>

a. Malignant cytologic features may be very subtle in some cases of mesotheliomas. This diagnosis may be overlooked and cellular findings may be misinterpreted as reactive/non-neoplastic. This is an extremely difficult distinction, because there is no marker that can differentiate benign mesothelial cells from malignant ones. In these cases the diagnosis of cancer should not be made unless appropriate clinical history including the review of previous effusions and/or strict cytologic criteria has been used in the interpretation of the cytologic material.

b. When the cells show cytomorphologic characteristics of malignancy, and at the same time they resemble mesothelial cells; in such cases the diagnosis of malignant mesothelioma can easily be made.

c. Mesotheliomas may show cytologic features indistinguishable from metastatic adenocarcinomas. The distinction here may be impossible on cytomorphologic grounds alone. In recent years, with availability of more specific markers such as calretinin and D2-40 this distinction is easy in majority of cases. Special attention should be made to the fact that this marker is not able to differentiate reactive mesothelial cells from those of malignant mesotheliomas. It should be noted that the Calretinin immunostaining in mesothelial cells is exclusively nuclear.

The following are diagnostic hints that may be useful in the distinction between mesotheliomas and adenocarcinomas:

1. When in clusters, cells of adenocarcinoma have a smooth border whereas mesothelioma cells usually have a "knobby surface".
2. Cytoplasmic vacuoles in adenocarcinomas are usually the result of mucin production, whereas non-mucinous vacuoles may be seen in degenerated mesothelial cells either benign or malignant.

3. Dense cytoplasm with small peripheral blebs suggests mesothelial origin; however, this may occasionally be seen in adenocarcinomas and squamous cell carcinomas.
4. The “cell within a cell” pattern that is frequently seen in mesotheliomas may also be seen in adenocarcinomas. In fact this finding may also be seen in non-neoplastic cells of mesothelial origin.
5. Binucleation is more commonly seen in mesotheliomas; however, it may also be seen in adenocarcinomas.
6. Cells in mesotheliomas are cuboidal or polygonal with a relatively constant N/C ratio, whereas adenocarcinoma cells are usually cuboidal or columnar with more evident nuclear molding and pleomorphism.
7. Extreme nuclear pleomorphism is more commonly seen in adenocarcinomas.
8. In cell block preparations the presence of papillary tissue fragments with fibrovascular core is more commonly seen in mesotheliomas, whereas true acinar formations are more common in adenocarcinomas.

The differential diagnosis between adenocarcinomas and malignant mesotheliomas can be facilitated by the use of immunocytochemistry.

Immunocytochemistry: Immunocytochemistry plays an important role in the diagnosis of malignancies in effusions. We use cellblock preparations or Papanicolaou-stained slides .The common indications for immunostaining in body cavity fluids are:

1. Differential diagnosis between reactive mesothelial cells and adenocarcinoma cells.
2. Distinction between mesotheliomas and adenocarcinomas.
3. Detection of primary site /subclassification of malignancies.

In our experience, the most common differential diagnosis requiring the use of immunocytochemistry, is the first one. In fact, more than 50% of immunocytochemical tests performed in our laboratory on cytologic samples are intended to resolve this differential diagnosis. There are two important facts that need to be recognized before immunocytochemical evaluation of effusions is attempted. First, both reactive and malignant mesothelial cells share the same immunostaining characteristics. Therefore, the differential diagnosis cannot be made on the basis of their immunostaining pattern. Thus, when the differential diagnosis is between malignant mesothelioma and reactive proliferation of mesothelial cells, the diagnosis should be based on cytomorphologic criteria only. Second, the pattern, frequency, or quantitative differences in expression of an antigen should not be the basis for separating of two different entities from each other. For example, relying on the pattern of cellular expression of cytokeratin and epithelial membrane antigen (EMA) in the differential diagnosis of malignant mesotheliomas and adenocarcinomas could lead to erroneous diagnostic conclusions. However, EMA, if interpreted with caution, can be of great value identifying malignant cells. Similarly, classifying cells in body cavity fluids as carcinoma

only because they react positively for EMA or B72.3 may lead to an erroneous diagnosis because these antigens are also expressed, although less frequently, by non-neoplastic cells.

Immunohistochemistry is an important diagnostic adjunct in the differential diagnosis between malignant mesothelioma and adenocarcinoma metastatic to the serous membranes. Most of the antibodies commonly used, however, recognize antigens expressed by the adenocarcinoma cells and absent from mesothelial cells and mesotheliomas. These markers are usually used in panels of two or more reagents to confirm or rule out the possible diagnosis of metastatic adenocarcinomas. Combined use of several of these antibodies increases the accuracy of the differential diagnosis. The most commonly used antibodies in our lab to differentiate mesothelioma from adenocarcinoma are CEA, EMA, Calretinin, D2-40, Estrogen Receptor, and TTF-1. Finally, if we are dealing with a non-epithelial neoplasm, other markers such as lymphoid markers, melanocytic markers and sarcoma markers among others are also utilized.

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SUPPLEMENT I

Routine Cytopreparatory Technique for Body Cavity Fluids

Accurate cytologic interpretation of body cavity fluids is dependent upon properly prepared cytologic slides. Poorly preserved cellular material may create difficulties in interpretation of these specimens. Since many problems arise when the fluid contains blood, large amounts of protein or inflammatory cells, we generally use the following simple and easily reproducible method for the preparation of body cavity fluids.

In our laboratory, most fluid specimens are processed fresh, with a final diagnosis reported within a few hours from the time the specimen was received. All specimens are submitted fresh, without added fixatives. If there is a delay in transportation of the fluid, we require refrigeration of the specimen. Bloody specimens are collected in containers with heparin, to prevent coagulation. Although some laboratories use filters such as the Millipore, Gelman, or Nucleopore for their fluid preparation, we have been using the Shandon Cytospin (Shandon, Inc. Pittsburgh, PA) to prepare all body cavity fluids. In our experience, evaluation of cytospin slides is less time consuming, and the results are essentially the same when compared to the filter preparations.

When preparing body fluids, we use a solution of Saccomano's fixative and an equal volume of physiologic saline (or water) to accomplish lysis of the red blood cells. This solution also helps in the preservation of the cells. On selected cases, we prepare cell blocks when we anticipate use of immunocytochemistry .However we have experienced the same sensitivity using previously Papanicolaou-stained slides.

Cytospin Preparation

1. Upon receipt of the specimen, we record the following information:
   a) amount of fluid received (in ml's)
   b) gross appearance of the specimen (e.g. bloody, cloudy, clear, xanthochromic, chylous, mucinous, watery, etc.)
2. The cells are gently re-suspended as they may have settled to the bottom of the container prior to processing. The specimen is placed in 2-6, 15ml centrifuge tubes and centrifuged at 1500 rpm for 2 minutes. *Note:* Specimens that are 2 ml. or less can be put directly in the cytospin sample chambers.
3. Glass slides are prepared while specimen is spinning. We prepare two slides (with exception of CSF specimens that we prepare 4 cytospins) with the patients name, accession number, and specimen type. All slides are coated with albumin. Extra slides may be made if special stains are requested.

4. The cytocentrifugal sample chamber is assembled and place in the cytocentrifuge.
5. The size of the cell button determines how much material is placed in the sample chamber. The supernatant is decanted and the specimen is re-suspended. If the cell button is large, usually only a drop or two is placed in each sample chamber. For small cell buttons, 3-6 drops can be added to each sample chamber.
6. The specimen is spun at 1000 rpm for 1 minute. After the centrifuge stops, the sample chamber is removed as quickly as possible as one unit. The slides are IMMEDIATELY placed in 95% isopropyl alcohol for fixation.
7. Slides can be stained after 5 minutes of fixation using the Papanicolaou staining technique. We routinely use a modified Papanicolaou staining method for our fluid specimens(see attached appendix).

SUPPLEMENT II
NON GYNECOLOGIC STAINING PROCEDURE
(Modified Papanicolaou Staining Technique)

All solutions are changed weekly. Water and heavily discolored alcohols are changed daily. Alcohols (50% and 70%) and NH4OH) are filtered daily after each run.

		MANUAL STAINING	**AUTOMATIC**
1.	80% Isopropyl alcohol	Count slowly to 10 (no dips)	10 seconds (no dips)
2.	70% Isopropyl alcohol	Count slowly to 10 (no dips)	10 seconds (no dips)
3.	50% Isopropyl alcohol	Count slowly to 10 (no dips)	10 seconds (no dips)
4.	H2O	Count slowly to 10 (no dips)	10 seconds (no dips)
5.	Harris Hematoxylin	30 seconds	30 seconds
6.	H2O	Count slowly to 10 (no dips)	10 seconds (no dips)
7.	H2O	Count slowly to 10 (no dips)	10 seconds (no dips)
8.	H2O	Count slowly to 10 (no dips)	10 seconds (no dips)
9.	50% Isopropyl alcohol	Count slowly to 10 (no dips)	10 seconds (no dips)
10.	NH4OH	1 minute	1 minute
11.	70% Isopropyl alcohol	Count slowly to 10 (no dips)	1 minute
12.	80% Isopropyl alcohol	Count slowly to 10 (no dips)	1 minute

13.	95% Isopropyl alcohol	Count slowly to 10 (no dips)	1 minute
14.	OG-6	15 seconds	15 seconds
15.	95% Isopropyl alcohol	Count slowly to 10 (no dips)	10 seconds (no dips)
16.	95% Isopropyl alcohol	Count slowly to 10 (no dips)	10 seconds (no dips)
17.	EA-65	1 1/4 minutes	1 1/4 minutes
18.	95% Isopropyl alcohol	Count slowly to 10 (no dips)	10 seconds (no dips)
19.	95% Isopropyl alcohol	Count slowly to 10 (no dips)	10 seconds (no dips)
20.	95% Isopropyl alcohol	Count slowly to 10 (no dips)	10 seconds (no dips)
21.	100% Isopropyl alcohol	Count slowly to 10 (no dips)	10 seconds (no dips)
22.	100% Isopropyl alcohol	Count slowly to 10 (no dips)	15 seconds
23.	100% Isopropyl alcohol & xylene	Count slowly to 10 (no dips)	15 seconds
24.	Xylene	Count slowly to 10 (no dips)	15 seconds
25.	Xylene	Coverslip slides	Coverslip slides

SUPPLEMENT III

UNIVERSITY OF MIAMI/JACKSON MEMORIAL MEDICAL CENTER
PATHOLOGY SPECIALTY SERVICES

IMMUNOPEROXIDASE PROCEDURE – STEPWISE: (Steps 12 through 17 are carried out in an autostainer in our lab.)

1. Cut paraffin sections to 3 microns.
2. Melt paraffin by placing slides in either a 58ºC oven for 5 minutes or preferably in a 37ºC oven overnight.
3. De-wax slides in xylene, 1 bath x 10 minutes.
4. Rehydrate slides in decreasing ethanol solutions, 1 minute each, 2 baths of absolute, 2 baths of 95%, 1 bath of 90%.
5. Block endogenous peroxidase with a solution of 6% Hydrogen Peroxide (H_2O_2) for 3 minutes.
6. Rinse slides by submerging in tap water for 1 minute.
7. Place rack in TBS (DAKO S1968) bath submerged for 1 minute.

8. Prepare target retrieval (TR): In a green staining dish measure 20 ml target retrieval (DAKO S1699) plus 180 ml DH_2O. Add DH_2O to steamer an turn it on. Place staining dish containing target retrieval solution inside steamer and let it heat for 20 minutes. TR solution should heat to 90ºC.
9. Take out staining dish from steamer and place slides inside dish (use gloves) and steam for 20 minutes.
10. After the steamer, let them cool down in same container for 20 minutes.
11. Place slides in PBS buffer at room temperature (in this step you can stop the technique and keep them in the buffer, from 2 minutes to 18 hours and then continue with the technique).
12. Biotin blocking system (DAKO X0590): The tissue sections are incubated with (1) Avidin Solution for 6 minutes. The avidin solution is then rinsed off and the slides are incubated with (2) Biotin Solution for 6 minutes. The biotin solution should be washed off before application of the first step of the staining procedure.
13. Add specific primary antibody to each slide, incubate for 30 minutes in a humidity chamber.
14. Place slides back in rack and submerge rack in TBS bath 2 minutes. Dry excess TBS off each slide, now add the linking solution (DAKO KO690 LSAB + Kit, biotinylated anti-mouse, anti-rabbit and anti-goat). Incubate for 22 minutes in humidity chamber.
15. Place slides back in rack and submerge rack in TBS bath 2 minutes. Then, DH_20 3 minutes and then TBS 2 minutes.
16. Dry excess TBS off slide. Add streptavidin-peroxidase-conjugate and incubate for 22 minutes in humidity chamber.
17. Submerge rack in TBS bath for 2 minutes. Make DAB (DAKO K3468) chromogen for next step (1 drop concentrated DAB per 1 ml substrate). Place rack in DAB solution for 10 minutes. Place slides into fresh TBS. Rinse slides for 4 minutes.
18. Dry slides and counterstain with hematoxylin (from 15 to 40 seconds).
    NOTE: For nuclear antigens: Dry excess TBS from slides and apply 1% Cupric Sulfate for 1 minute. Rinse slides in tap water for 2 minutes. Place slides in 0.2% fast green for 1 or 2 seconds.
19. Dehydrate through gradient alcohols, Clean in xylene and coverslip.

Madrid. 25 de Abril de 2013.

XXVI CONGRESO NACIONAL DE LA SEAP – IAP. XXI CONGRESO DE LA SEC. II CONGRESO DE LA SEPAF. CADIZ – 22 -24 DE MAYO.2013.

RESUMEN DE COMUNICACIÓN. CURSO DE LIQUIDOS ORGANICOS.

TITULO : " MARCADORES DE PROLIFERACION EN LA CITOLOGIA URINARIA DE RUTINA Y EL CARCINOMA UROTELIAL. ESTUDIO EN 30 PACIENTES Y 10 AÑOS DE SEGUIMIENTO ".

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RESUMEN DE LA COMUNICACION :

Las neoplasias uroteliales representan entre el 80 y el 90 % de los tumores del tracto urinario. Los inmunomarcadores MIB-1 (Ki-67) y Bcl-2, junto con la citoqueratina CK-20, marcan diferencias en cuanto a la progresión y el pronóstico del Carcinoma Urotelial. En el estudio actual valoramos estos marcadores sobre la Citología Urinaria en 30 pacientes, procesadas en citología líquida – Thin – Prep. (Hologic), en las que tras la tinción de Papanicolaou se realizan posteriormente estas inmunodeterminaciones. El objetivo es valorar la correlación entre la Progresión, el Grado del Tumor y la Estadificación en estos pacientes, utilizando estos marcadores sobre la citología urinaria de rutina, para separar los de alto riesgo, con mayor posibilidad de recidiva y que puedan beneficiarse de la Terapia adyuvante. Nuestro estudio incluye a 30 pacientes, comprendidos entre los 53 y 90 años de edad, seguidos en un periodo de 10 años con Carcinoma Urotelial, la mayoría en estadío T1 inicial y con distintos grados de diferenciación histológica. Especialmente se evalúan las posibles progresiones de un Bajo a un Alto Grado utilizando estos inmunomarcadores en la citología de rutina.

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