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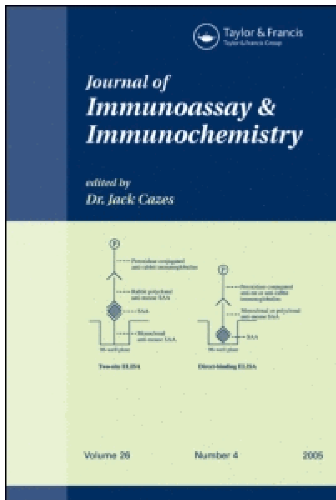

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A Quantitative Nitrocellulose Enzyme Immunoassay and its Application to the Screening of Hybrbidomas for the Detection of Uncharacterized Tumor-Associated Antigens in Sera of Cancer Patients

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A QUANTITATIVE NITROCELLULOSE ENZYME IMMUNOASSAY AND ITS APPLICATION TO THE SCREENING OF HYBRBIDOMAS FOR THE DETECTION OF UNCHARACTERIZED TUMOR-ASSOCIATED ANTIGENS IN SERA OF CANCER PATIENTS

(KEY WORDS: ELISA, Monoclonal antibody, Hybridoma, Tumor associated antigens, Cancer patient's serum.)

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ABSTRACT

An enzyme-linked immunosorbent assay on nitrocellulose based microtiter plates for the detection of uncharacterized tumor associated antigens in squamous cell carcinoma and adenocarcinoma cancer patients' sera is described. Nitrocellulose microtiter plates are more sensitive than the plastic plates of polystyrene and polyvinyl chloride for the detection of antigens in serum. Monoclonal antibodies were selected for their net reactivities toward cancer patients' sera as compared to nomal sera. Sera from benign liver and kidney disease patients and activated human peripheral blood leukocyte supernatant were used to reduce potential false positives toward inflammatory and benign diseases. Using this system, fourteen antibodies were selected out of over eight hundred antibodies for their potential serodiagnostic application.

INTRODUCTION

One of the most critical steps in the complex task of establishing monoclonal antibodies (MAb) is probably the effective screening of the hundreds of MAbs secreted by the hybridomas in order to identify the useful ones for a specific application. Much has been

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reported about the immunization, selection of fusion partner cell lines, culturing and growth of hybridomas in selective medium and production of antibody *in vivo* and *in vitro* (1, 2, 3, 4, 5, 6, 7, 8, 9, 10). However, relatively little has been reported regarding the design of a simple and efficient screening method that has the capability to test hundreds of hybridoma culture supernatant fluids against the desired, yet uncharacterized tumor associated antigens in a complex milieu such as serum and other body fluids.

It is often necessary to select MAbs for their specific reactivities to a set of predetermined requirements, e.g., the selection of MAbs which can detect tumor associated antigens in the sera of cancer patients before the identity and nature of the antigens are even known. The only important parameter at the early stage of the selection process is that the candidate MAb demonstrates that there is an antigen concentration difference in the sera of cancer patients as compared to those of normal and benign individuals, so that the MAb can functionally be used to distinguish the sera of cancer patients from those derived from normal or benign populations. The characteristics of the antigen recognized by the MAb are not crucial, at least not at this stage of the experiment. The most frequently used methods for screening MAbs produced by hybridomas are based on the observation of Catt and Tregear (11) that proteins tend to bind tightly to plastic surfaces. Because soluble antigens can easily be immobilized on the plastic 96-well microtiter plates, the method has been very popular. However, we have found the sensitivity on the plastic solid support is not adequate for the detection of low concentration antigens in complex body fluids such as serum, sputum, bronchial washes and urine, or *in vitro* cultured cell line supernatants. The search for MAbs which have the capabilities to identify tumor associated antigens that are shed by the cancer cells and circulated into body fluids such as serum is difficult. The antigens are mostly unknown in nature, not available in pure form for screening experiments, are present generally in low concentrations, and they may also be found in non-cancerous inflammatory and benign conditions. In an attempt to use such fluids as the antigen sources to select useful MAbs, one

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can not ignore the presence of normal yet irrelevant molecules which are in abundance in these fluids. Therefore, the screening method has to address specifically the inherent problems associated with these body fluids.

In the present report, we describe a screening method which uses a nitrocellulose based microtiter plate enzyme-linked immunosorbent assay (ELISA) in combination with the computer program Lotus 123 (Lotus Development Corporation, Cambridge, Massachusett, USA) for data management to screen useful MAbs.

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MATERIALS AND METHODS

Materials

Ninety-six well polystyrene (Immulon) and flexible round-bottom polyvinyl chloride (PVC) microtiter plates were obtained from Dynatech Laboratories. Nitrocellulose based microtiter plates (Millititer HA) were obtained from Millpore Corporation. Normal goat serum was obtained from Vector Laboratories. Goat anti-mouse IgG (gamma chain specific) horseradish peroxidase (HRPO) conjugate was obtained from Southern Biotechnology Association, Inc. Goat anti-mouse IgM (mu chain specific) HRPO conjugate was obtained from Tago, Inc. Tween 20, Ficoll-hypaque, concanavalin A (Con A), pokeweed mitogen (PWM), heparin and o-phenylenediamine dihydrochloride (OPD) were obtained from Sigma Chemical Company. Human type A and B serum and phytohemagglutinin (PHA) were purchased from Difco Laboratories (Detroit, MI).

Human Serum

Sera from cancer patients with squamous cell carcinoma (SCC) of the head and neck, lung and cervix; adenocarcinoma (Adeno) of the colon, breast and ovary; and sera from

subjects with non-cancerous conditions such as liver cirrhosis and renal failure were collected under Institutional Review Board Approval at Stanford University Medical School and University of California, San Francisco Medical School. Sera from healthy normal blood donors were obtained from a local blood bank. All sera were stored in aliquots at -20°C. The SCC sera, adenocarcinoma sera, liver and kidney disease sera were pooled separately and used in ELISA as antigen sources to screen MAbs.

Activated Human Peripheral Blood Leukocyte (PBL) Supernatant

Three bags of fresh human buffy coats from different individuals purchased from a local blood bank were used to obtained mononuclear cells by centrifugation over Ficoll-hypaque gradient. The buffy large cells were washed in phosphate buffered saline and counted in a hemacytometer. Cell pellets were diluted to 5 x10^{6} cells/ml in RPMI 1640 medium containing human A and B serum (1.0%), L-glutamate (1.0%), penicillin (100 units/ml), streptomycin (100 ug/ml), and 2-mercaptoethanol (50 umol); this solution is called PBL culturing medium. The diluted cells were then stimulated by adding the following mitogens; 1 ug/ml Con A, 1.0% PHA and 3ug/ml PWM. The cells and mitogens were incubated in T-75 tissue culture flasks at 37°C in a 5% CO_2 incubator for 48 hr. After incubation, cells were spun at 1500 rpm for 20 min. and the supernatants were collected. The resulting supernatant, which contains molecules such as Interleukin 1, 2, 3 and 4; other B cell growth factors; alpha and gamma interferons; macrophage activating factors and prostaglandins, etc. that are associated with various inflammatory conditions was used to eliminate MAbs which are recognizing these antigens associated with inflamatory conditions *in vivo* in order to reduce the potential false positivity of the selected MAbs to recognize tumor associated antigens.

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Monoclonal Antibodies

The MAbs used in this study have been developed at InTek Diagnostics. The method used to produce these MAbs for tumor associated antigens has been previously described (12). Briefly, hybridomas were produced according to the method of Fazekas de St. Groth and Scheidegger (2). Cancer cells derived from surgically or biopsy removed cancer tissues or mixtures of human cancer cell lines were used as the immunogens. Hyperimmunized BALB/c splenocytes were fused with non-secreting mouse myeloma cell line Sp2/0. Antibodies were initially screened for immunofluoresence and immunoperoxidase staining on frozen sections of normal and tumor tissues.

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Conditions for ELISA on Plastic and Nitrocellulose Based Microtiter Plates

The conditions for ELISA are same for the plastic and nitrocellulose microtiter plates except the nitrocellulose bottomed microtiter plates need to be washed once before use with 0.01 M phosphate, 0.16 M NaCl, pH 7.2 (PBS) to wet the wells, and the solution needs to be filtered through with a Millipore vaccum apparatus. The ELISA steps were performed as follows: 1) add 50 ul per well of pooled SCC serum, adenocarcinoma serum, benign serum, activated PBL supernatant or human normal serum as antigen source. Incubate microtiter plates at 4°C overnight. 2) Add 100 ul of 10% normal goat serum in PBS for 1 hr at room temperature. 3) After washing three times with PBS, add 50 ul of the primary MAb to be screened (either as hybridoma supernatant or for purified MAb, at a concentration of 10 ug/ml in PBS with 10% normal goat serum) at room temperature overnight. 4) After washing three times with PBS-0.05% Tween-20, add 50 ul of HRPO-conjugated anti-mouse IgG or anti-mouse IgM in 1:1000 dilution at room temperature for one and half hrs. 5) after washing three times with PBS-Tween, add 100 ul of OPD (3 mg/ml) in phosphate-citrate buffer (25 mmol/ml, pH 5.15) containing H_2O_2 (0.2g/l), incubate at room temperature for 15 min in dark. 6) Stop reaction by adding 25 ul of 1.25 M sulfuric acid and read the absorbance at 492

nm ($A_{492\ nm}$) in a microtiter plate spectrophotometer. Substrate solution in nitrocellulose bottomed microtiter plates was first transferred to a polystyrene microtiter plate with a multi-channel pipet before the absorbance was measured.

Data Analysis

Lotus 123 program was used in a double subtraction mode as indicated in the following:

First, the absorbance value of each individual well which contains cancer patients' sera, benign patient's sera or pooled normal sera as the screening antigen is subtracted by the background absorbance value of a control well which has no primary MAb but with HRPO-conjugated anti-mouse IgG or IgM and H_2O_2/OPD were added to the well for the ELISA procedure. The derived absorbance value is then subjected to a secondary subtraction by the absorbance values derived from the activated PBL supernatant, benign or normal human serum as the screening antigens. In summary:

1) A MAb's net reactivity towards potential inflamatory conditions, i.e., :

Net PBL A_{492nm} = A_{492nm} of PBL Supernatant - A_{492nm} of PBL medium ;

2) A MAb's net reactivity towards potential benign conditions, i.e., :

Net Benign A_{492nm} = A_{492nm} of pooled benign serum - A_{492nm} of pooled normal sera;

3) A MAb's net reactivity towards SCC associated antigen, i.e.,:

Net SCC A_{492nm} = A_{492nm} of SCC serum - A_{492nm} of pooled normal sera;

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4) A MAb's net reactivity towards adenocarcinoma associated antigen, i.e.,:

Net Adeno A_{492nm} = A_{492nm} of adenocarcinoma patients serum -

$A_{492\ nm}$ of pooled normal sera.

The reproducibility of these absorbance values are indicated as the coefficient of variance.

A MAb is selected for its potential serodagnostic application when it has a net positive SCC and / or adenocarcinoma absorbance with a low net absorbances derived from activated PBL supernatant and benign diseases pooled sera. The larger the ratio of the SCC or adenocarcinoma absorbance over either the PBL or benign sera absorbance, the more likely the MAb will be specific towards cancer.

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RESULTS

Sensitivity Comparison of Antigen Detection in Serum by ELISA Using Polystyrene, PVC and Nitrocellulose Based Microtiter Plates.

In order to compare the feasibility of detecting low concentrations of antigen in serum by different types of microtiter plates in an ELISA method, purified mouse IgM was used as the simulating antigen and was attempted to be detected by the ELISA procedure in various types of microtiter plates. Various concentrations of mouse IgM from 0 to 10 ug/ml were added to either PBS or to the pooled normal human serum and immobilized onto the wells of microtiter plates. The ELISA was carried out according to the method described. Since the mouse IgM was the designated antigen and it was immobilized onto the plates, the HRPO-conjugated goat anti-mouse IgM antiserum was then added directly into the wells without the addition of a primary antibody. The dose response titration curves derived from the polystyrene, PVC and nitrocellulose microtiter plates are shown in Figure 1. It is clear that the mouse IgM was easily detectable in all three types of microtiter plates tested when it was

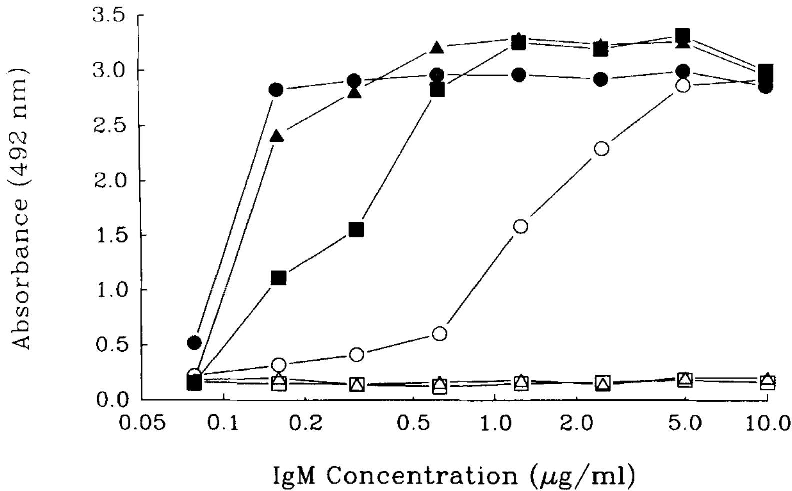

Figure 1. ELISA dose response curves for the detection of mouse IgM using polystyrene microtiter plate (■ , IgM in PBS), (□ , IgM in human serum); polyvinyl microtiter plates (▲ , IgM in PBS), (△ , IgM in human serum); and nitrocellulose based microtiter plates (● , IgM in PBS), (○, IgM in human serum).

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diluted in PBS (the top three curves). However, the detection sensitivity is quite different when the mouse IgM is diluted in normal human serum. At an IgM concentration of 10 ug/ml, no IgM was detectable by either the polystyrene or the PVC microtiter plates (the bottom two curves). In contrast, the IgM in the nitrocellulose wells can be detected at a concentration of 0.625 ug/ml (the middle curve). This represents at least a sixteen (10/0.625=16) fold increase in sensitivity improvement. These data indicated that it is probably feasible to detect tumor associated antigens that are glycoprotein macromolecules in nature like IgM in serum by this system. It is most unlikely to have an undefined tumor associated antigen present in serum at a concentration of much greater than 10 ug/ml. At least it is not likely when the tumor burden is still relatively small and restricted.

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Application of the Nitrocellulose ELISA Method for the Screening of MAbs

Over eight hundred MAbs have been screened by this nitrocellulose ELISA method using activated PBL supernatant, sera from benign liver and kidney disease patients, SCC and adenocarcinoma cancer patients, and normal healthy individuals as the antigen sources for screening. Among these MAbs, some did not demonstrate reactivities to any of the antigen sources, some had net positive reactivities toward only the activated PBL supernatant or pooled benign sera, some MAbs demonstrated reactivities for both cancer patients sera, activated PBL supernatants and benign antigen sources. Few MAbs demonstrated a net positive reactivity toward either the SCC and/or adenocarcinoma cancer patients' sera.

Fourteen MAbs That Have the Potential for the Detection of SCC and Adenocarcinoma Associated Antigens

By using the nitrocellulose ELISA method with various antigen sources and Lotus 123 data reduction, a total of fourteen MAbs which have demonstrated a net positive reactivities for either SCC and/or adenocarcinoma patients' sera are presented here. Table 1 indicates the immunogens used for the generation of these MAbs, the antibody isotypes and their immunohistological reactivities toward SCC and adenocarcinoma cancer tissues by immunoperoxidase staining. The net A_{492nm} values derived from various antigen sources tested are shown in Table 2. As indicated by the coefficient of variation (CV) data in Table 2, the method is reproducible. Generally, the CV is within 10% range. Among these fourteen MAbs, MAb A, B, C and F have net positive reactivities against SCC cancer patients' sera; MAb H, I, J and K have net positive reactivities against adenocarcinoma cancer patients' sera; while MAb D, E, G, L, M and N have a net positive reactivities against both SCC and adenocarcinoma cancer patients' sera. There is no consistent corrleation between a MAb's immunohistological staining reactivity for cancer tissues and its reactivity against a tumor associated antigen in serum. No paired study of tissues and serum derived from a same cancer

TABLE 1

Description of the MAbs that Demonstrated a Net Positive Reactivity Towards SCC or Adenocarcinoma Cancer (ACA) Patients' Sera

MAb	Immunogen	Isotype	Immunoperoxidase staining reactivity on cancer tissues
A	SCC tissue from human tongue	IgM	SCC
B	SCC cell lines from human head & neck region	IgM	SCC
C	same as B	IgM	SCC
D	same as B	IgM	SCC
E	sera from SCC patients	IgM	neither SCC nor ACA
F	same as E	IgM	neither SCC nor ACA
G	dysplastic cells from human cervix	IgG 1	SCC
H	ACA tissues from the breast, colon and ovary	IgM	ACA
I	same as H	IgM	ACA
J	same as H	IgM	ACA
K	same as H	IgM	ACA
L	SCC cell lines from human head & neck region	IgM	SCC & ACA
M	ACA cell lines from female gynaeco-logical cancers	IgM	SCC & ACA
N	ACA tissue from human breast cancer	IgM	ACA

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TABLE 2

Detection of Potential Tumor Associated Antigens in Sera of Cancer Patients by the Fourteen MAbs in the Nitrocellulose ELISA

MAb	Net PBL A_{492nm}	Net Benign A_{492nm}	Net SCC A_{492nm}	Net Adeno A_{492nm}	Net SCC/Net PBL* or Net ACA/Net PBL*	Net SCC/Net Benign* or Net ACA/Net Benign*
A	-0.05 (8.0%)**	-0.32 (6.3%)	0.12 (4.6%)	0.01 (0.9%)	12	12
B	-0.10 (5.0%)	-0.16 (2.5%)	0.25 (1.8%)	-0.04 (10.8%)	25	25
C	0.06 (0.7%)	-0.16 (2.9%)	0.34 (11.4%)	0.06 (14.6%)	5.7	34
D	-0.11 (8.3%)	0.03 (2.8%)	0.33 (4.1%)	0.14 (2.3%)	33	11
E	0.09 (5.9%)	0.03 (15%)	0.34 (0.9%)	0.01 (4.4%)	3.1	11
F	0.11 (1.1%)	0.03 (0.6%)	0.34 (1.0%)	0.01 (6.4%)	3.1	11
G	0.09 (4.3%)	0.03 (2.5%)	0.23 (2.8%)	0.26 (8.6%)	2.9	8.7
H	-0.13 (0.1%)	-0.35 (8.6%)	-0.01 (1.8%)	0.14 (7.5%)	14	14
I	-0.30 (1.2%)	-0.29 (3.0%)	0.03 (2.5%)	0.31 (0.4%)	31	31
J	-0.19 (0.78%)	-0.05 (1.1%)	0.05 (0.4%)	0.40 (6.3%)	40	40
K	-0.13 (3.8%)	-0.31 (2.9%)	-0.08 (4.9%)	0.45 (6.9%)	45	45
L	0.09 (0.2%)	0.11 (1.5%)	0.25 (0.8%)	0.32 (4.2%)	3.6	2.9
M	0.95 (8.3%)	-0.05 (10.1%)	0.20 (2.3%)	0.45 (7.7%)	9.0	45
N	0.03 (2.2%)	-0.08 (3.1%)	0.28 (8.2%)	0.71 (4.3%)	24	71

*Negative Values in the Net PBL A_{492nm} and Net Benign A_{492nm} were treated as 0.01 to derive the ratios.

**The reproducibility of each absorbance value is provided by % coefficient of variation in the parenthesis.

patient was carried out in this study. All fourteen MAbs have weak or net negative reactivities toward activated PBL supernatant and sera derived from benign diseases. Although the nature and characteristics of these antigens in the cancer patients' sera are still unknown, these MAbs have already demonstrated their capabilities to distinguish cancer from benign and inflammatory conditions.

DISCUSSION

The enzyme immunoassay presented here is a simple, quantitative and reproducible method for the selection of MAbs which may be useful for the serodiagnosis of cancer. We selected nitrocellulose as the solid support because of its reported higher binding capacity for proteins (13, 14, 15, 16, 17). Applying nitrocellulose as the solid support in a microtiter format has enabled the assay to be carried out beyond visual qualitative detection to a quantitative mode. By using mouse IgM as a model to simulate the uncharacterized tumor associated antigen molecule in serum, our data further confirmed that nitrocellulose is more sensitive than plastic supports for the detection of glycoprotein macromolecules in a body fluid such as serum.

Most of the reported tumor associated antigens are macromolecules containing protein or glycoprotein moeity (18). The nitrocellulose ELISA screening method described here should be useful for the detection of some tumor associated antigens which are either proteins, glycoproteins, enzymes or mucins that contain polypeptides or carbohydrate component. The method is especially suitable for the selection of MAbs to detect the antigens which are defined by monoclonal antibodies, i.e., the nature, identity and characteristics of the antigen is unknown prior to the corresponding MAb is produced and well studied. The majority of the widely used tumor markers such as carcinoembryonic antigen and alpha fetoprotein are not defined by MAb; neither are they tumor specific. Their presence and structural characterization are known well before the hybridoma technology was invented.

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The presence of these antigens in benign liver and kidney diseases and other inflamatory conditions such as pancreatitis, ulcerative colitis, diverticulitis and hepatitis, etc., are well known (19). Therefore, the clinical utilities of these tumor markers are restricted mainly to the post-diagnostic monitoring use. We have described a systematic approach in this report to reduce the false positive potentials of the selected MAbs. We are currently using the described system to select MAbs for the development of serodiagnostic tests for the management of SCC and adenocarcinoma cancer patients.

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