Use of Yeast-Secreted *In vivo* Biotinylated Recombinant Antibodies (Biobodies) in Bead-Based ELISA

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Abstract

Purpose: To measure circulating antigens, sandwich ELISA assays require two complementary affinity reagents. Mouse monoclonal antibodies (mAb) and polyclonal antibodies (pAb) are commonly used, but because their production is lengthy and costly, recombinant antibodies are emerging as an attractive alternative.

Experimental Design: We developed a new class of recombinant antibodies called biobodies (Bb) and compared them to mAb for use in serodiagnosis. Bbs were secreted biotinylated *in vivo* by diploid yeast and used as affinity reagents after Ni purification. Bead-based assays for HE4 and mesothelin were developed using Bbs in combination with pAbs (Bb/pAb assays). To assess precision, reproducibility studies were done using four runs of 16 replicates at six analyte levels for each marker. Pearson correlations and receiver-operator characteristic analyses were done in 214 patient serum samples to directly compare the Bb/pAb assays to mAb assays. Diagnostic performance of the Bb/pAb assay was further assessed in an expanded set of 336 ovarian cancer cases and controls.

Results: On average across analyte levels, Bb/pAb assays yielded within-run and between-run coefficients of variations of 11.7 and 23.8, respectively, for HE4 and 14.0 and 14.5, respectively, for mesothelin. In the subset (n = 214), Pearson correlations of 0.95 for HE4 and 0.92 for mesothelin were observed between mAb and Bb/pAb assays. The area under the curves for the mAb and Bb/pAb assays were not significantly different for HE4 (0.88 and 0.84, respectively; P = 0.20) or mesothelin (0.74 and 0.72, respectively; P = 0.38).

Conclusion: Yeast-secreted Bbs can be used reliably in cost-effective yet highly sensitive bead – based assays for use in large validation studies.

Large-scale screening studies to evaluate candidate ovarian cancer early detection biomarkers with immunoassays are challenged both by limitations in the quantity of patient sample required and by the need for large amounts of expensive and work-intensive affinity reagents. To overcome the first limitation, we adapted double-determinant ELISA assays to a bead-based platform that uses spectrally discrete polystyrene beads or microspheres instead of flat surfaces to immobilize the capture antibody (1, 2). Each antibody-coupled microsphere captures soluble antigens that are then detected with biotinylated antibody and phycoerythrin-conjugated streptavidin. A reading system based on flow cytometry (Bio-Plex Protein Array System, Bio-Rad) measures the fluorescent signals generated by streptavidin-phycoerythrin and the microspheres when they are close to each other after formation of antigen/antibody complexes. The data are reported as median fluorescence intensity. We developed bead-based assays with available mouse monoclonal antibodies to detect CA125 and HE4 (3) and two biomarkers for ovarian carcinoma (4 – 7), and we showed that the CA125 and HE4 assays did comparably with the standard CA125II RIA and the HE4 ELISA, respectively, while requiring only 15 μL of serum.

To address the second limitation, we simplified the generation and reduced the cost of producing affinity reagents by developing a new class of reagents known as biobodies (Bbs; ref. 8). Bbs are recombinant antibodies secreted by diploid yeast as HIS-tagged, *in vivo* biotinylated proteins. Diploid yeast result from the fusion of two haploid yeast of opposite mating type. In our system, one haploid yeast carries a cDNA encoding an antibody recognition sequence fused at

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the NH_2 terminus to the α-prepro secretion leader and at the COOH terminus to a His_6 tag, a prolinker of the IgA1 hinge and a biotin acceptor site; the other yeast carries a cDNA encoding an *Escherichia coli* biotin ligase (BirA) fused to the yeast *KEX2* golgi localization sequences. BirA can then catalyze biotin transfer to the fusion protein as it transits the yeast secretory compartment. Bbs can bind to labeled streptavidin and streptavidin-coated surfaces while still in yeast culture supernatant or after a simple Ni purification. This makes supplementary steps of chemical biotinylation unnecessary, thereby reducing preparation time relative to hybridoma supernatant or ascites purification. In addition and importantly, *in vivo* biotinylation preserves the recognition function of recombinant antibodies through a targeted biotinylation (8).

We previously generated Bbs against HE4 that showed specificity and sensitivity by ELISA assays, flow cytometry analysis, and Western blots before any maturation. The Bb dissociation K_s, as measured by surface plasmon resonance sensor, were of $K_d = 4.8 \times 10^{-9}$ mol/L and $K_d = 5.1 \times 10^{-9}$ mol/L before and after purification, respectively (8). We also developed and validated anti-mesothelin Bbs of high affinity that could detect both membrane-bound and soluble forms of mesothelin (9). Mesothelin is an epithelial marker highly expressed by cancer cells from diverse origins, including ovarian and pancreatic adenocarcinomas and mesotheliomas (10, 11). Elevated serum mesothelin levels have been reported in ovarian cancer (12–15) and mesothelioma (16, 17). In mesothelioma patients, mesothelin serum levels correlate with tumor size and increase during tumor progression (16).

Here, we describe the development, validation, and diagnostic performance of bead-based assays using Bbs and polyclonal antibodies (pAb) for the measurement of serum HE4 and mesothelin in ovarian carcinoma patients and controls obtained through the Pacific Ovarian Cancer Research Consortium (POCRC). We assessed the precision of the assays in reproducibility experiments using 16 replicates of six analyte levels in each of four runs (plates), yielding 64 replicates of each level of the two analytes. We assessed diagnostic performance of the novel Bb/pAb assays in 336 samples; a subset of these sera ($n = 214$) was used to assess the validity of the assays and to compare their diagnostic accuracy in serous ovarian cancer. We show that Bb/pAb assays are reproducible, correlate highly with assays using mAbs (mAb assays), and perform as well as the mAb assays in distinguishing between case and control sera. This system will allow us to develop cost-effective yet highly sensitive and reliable reagents for use in large population–based validation studies for evaluation of novel markers discovered through emerging proteomics technologies.

Materials and Methods

Antibodies and secondary reagents. Anti-HE4 (8) and anti-mesothelin (9) Bbs were Ni-purified from yeast culture supernatants as previously described (8) and dialyzed against PBS (Fisher BioReagents). The anti-mesothelin pAb was acquired from R&D Systems, and the anti-HE4 pAb was developed as described previously (18). Briefly, HE4-specific pAb were raised by immunizing rabbits with a glutathione *S*-transferase (GST) fusion protein composed of the mature form of HE4 (amino acids 31-125) and GST. Affinity purified antibodies were generated by adsorption of the crude antisera to a GST affinity column (Pierce Biotechnology, Inc.) to remove all the GST antibodies. The GST antibody-depleted serum was then affinity-purified by passing it over a GST-HE4 column generated using an AminoLink Coupling Gel column (Pierce Biotechnology, Inc.). Anti-mesothelin 4H3 and ovcar569 mAbs, and anti-HE4 2H5 and 3D8 mAbs were kind gifts from Dr. Ingegerd Hellström. Horseradish peroxidase–conjugated antihuman immunoglobulin and antimouse immunoglobulin antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. Bbs and biotinylated mAbs were detected with streptavidin-phycoerythrin (BD PharMingen) or PhycoLink Streptavidin-R-Phycoerythrins PJ31S, PJ35S, PJLS, PH37S, PH39S, and PJ33S (Prozyme). Antibodies were dialyzed against PBS when needed. Carboxy-coated microspheres (Bio-Rad Laboratories) were covalently coupled with various concentrations (0.2, 1, 5, 10, 20, and 50 μg/mL) of pAb according to manufacturer's instructions.

Biobody and assay development overview. We previously isolated anti-HE4 and anti-mesothelin recognition sequences encoding antigen-specific scFv from a yeast-display scFv library (19) using HEK293F cell–secreted HE4 protein fused to an immunoglobulin domain (HE4-Ig; ref. 3) and a yeast-secreted mesothelin recombinant protein (meso-7; ref. 9), respectively (Fig. 1). Briefly, a yeast-display scFv library was enriched for scFv binding to HE4 (8) or mesothelin (9), called "marker," by two magnetic enrichments (Fig. 1A) and three fluorescent cell sortings (Fig. 1B). ScFv selected for HE4 binding but also bound nonspecifically to meso-Ig (8) were removed by magnetic depletion (Fig. 1C). The recognition sequences of the marker-specific yeast-display scFv were PCR amplified and cotransfected into yeast with the vector pTOR2 (8) for cloning by gap repair (Fig. 1D). Transformed yeast colonies were grown in 1 mL of medium in 2 mL 96-well plates (Fig. 1E) and induced in presence of galactose to produce secreted marker-specific, tagged scFv. Yeast supernatants were high-throughput purified as described in (ref. 9; Fig. 1F) and analyzed by capture ELISA (Fig. 1G) for specific binding to the marker. ScFv selected for mesothelin binding but also bound nonspecifically to CA125 repeat domain (9) were eliminated by ELISA screening (Fig. 1G). Yeast that secreted marker-specific scFv were then mated with yeast that produced a golgi-localized biotin ligase (ref. 8; Fig. 1H) to generate diploid to secrete marker-specific Bbs. Diploid yeast were finally grown in liquid medium (Fig. 1I) and induced to produce secreted Bbs that were Ni-purified (Fig. 1J). Ni-purified Bbs were combined with PE-labeled streptavidin and tested for their sensitivity, specificity (8, 9), and ability to best complement pAb for the detection of serum antigens in double-determinant assays ("sandwich" ELISA or bead-based assay; Fig. 1K).

Production and validation by detection ELISA of yeast-secreted HE4 and mesothelin recombinant proteins. HE4 cDNA (4) was reverse-transcribed and amplified from primary ovarian cancer tissue using the primers that exclude the leader sequence, forward 5′-gattataaagatgacgataaaggtggtggtggttctgctagc accgatgcagagaaacccg-3′ and reverse 5′-gggttagggataggcttaccctgttgttctagaattccgaatttgggtgtggtgcagg-3′. The exclusion of the leader sequence was necessary to obtain a high yield of yeast-secreted protein (data not shown). The 350-bp product was then cloned into the pTOR2 plasmid by gap repair via cotransformation into the YVH10 secretion yeast (EasyComp Transformation kit, Invitrogen). Transformed yeast secreted a His-tagged HE4 protein referred to in the rest of the study as HE4y. After mating with pTOR BIR-carrying yeast as described in (ref. 8), the resulting diploid secreted *in vivo* biotinylated HE4y (b-HE4y) was validated by detection ELISA (Fig. 2A) using anti-HE4 3D8 or 2H5 mAbs. ELISA immunoassays were done in Streptavidin Immobilizer plates (Nunc) coated with biotinylated proteins diluted in PBST (1× PBS plus 0.05% Tween 20; Sigma-Aldrich). Incubations and washes were done with PBST at room temperature with gentle agitation. Colorimetric signals were generated with 50 μL TMB One Solution (Promega), stopped with 50 μL 1N H_2SO_4 (Acros Organics USA), and read at 450 nm on a Spectra Max 250 (Molecular Devices). Mesothelin antigen production in yeast was described previously (9) using similar methods.

Study population and serum samples. Assay precision was assessed in reproducibility experiments using pooled sera from ovarian cases and controls obtained from the POCRC repository. Case-pool serum was formed by combining 1 mL of serum from each of 50 patients

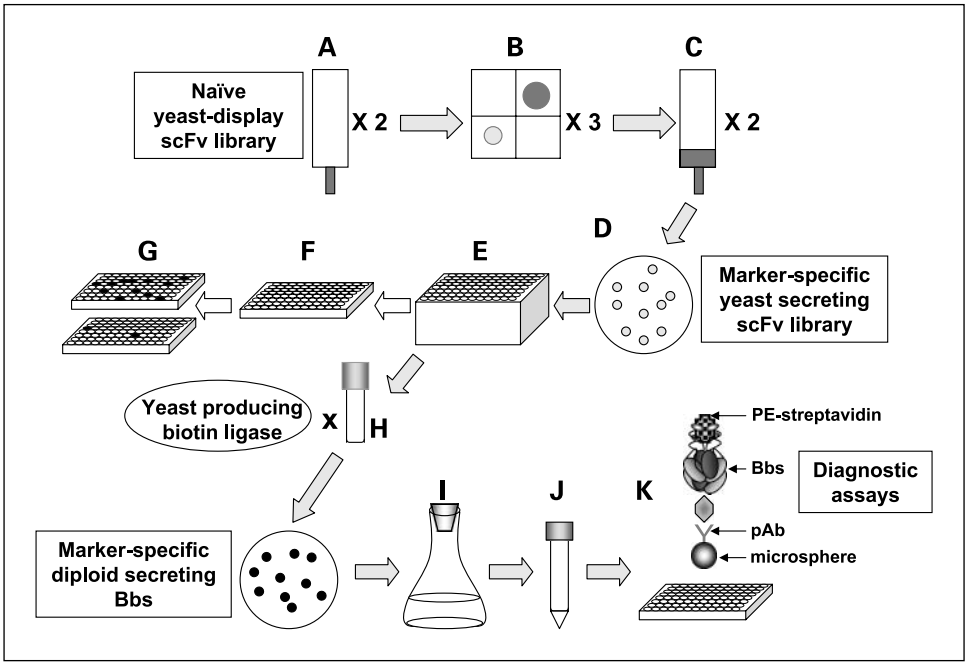

Fig. 1. Method overview. *A* and *B,* enrichment of a yeast-display scFv library for scFv binding to markers by two magnetic enrichments (*A*) and three fluorescent cell sortings (*B*). *C,* depletion of the enriched library for cross-reactive scFv. *D,* PCR amplification of marker-specific recognition sequences and yeast cotransformation with the vector pTOR2 for cloning by gap repair. *E,* growth and induction of yeast-secreting scFv in liquid medium. *F,* high-throughput Ni purification of secreted scFv. *G,* analysis for marker specificity by capture ELISA. *H,* mating of yeast secreting marker-specific scFv with yeast producing biotin ligase. *I,* growth and induction of diploid yeast to secrete marker-specific Bbs. *J,* large-scale Ni purification to obtain reagents for double-determinant assays. *K,* reagent ready for diagnostic tests, such as bead-based assays.

diagnosed with late stage ovarian cancer; control-pool serum was made from serum collected from seven healthy female volunteers. Intermediate pools of serum were created by serial dilution of the case pool with control pool by factors of two, yielding four pools with 1:1, 1:3, 1:7, and 1:15 ratios of the case-pool serum and control-pool serum (notation; parts case pool/parts control pool). The resulting six sera dilutions include high, low, and intermediate levels of the two analytes. A reproducibility experiment was conducted for each analyte using 16 replicates of each serum dilution in each of four runs (plates), providing a total of 64 replicates for each analyte level. Replicates were randomized onto the 96-well plates, and each of three operators ran at least one plate for each analyte.

Diagnostic performance of the Bb/pAb assays for HE4 and mesothelin was evaluated in a set of 336 serum samples, including 116 cases (73 serous and 43 nonserous) and 220 controls, randomly selected from the POCRC repository. The validity (correlation between) and relative diagnostic performance of the Bb/pAb and mAb assays for HE4 and mesothelin were assessed in a subset of 214 samples (71 cases and 143 controls) that was characterized for both the mAb and the Bb/pAb assays for both HE4 and mesothelin.

Cases were defined as having invasive epithelial ovarian carcinoma confirmed by standardized review of medical records and pathologist examination of paraffin-embedded tissue. Each set included serous, mucinous, endometrioid, clear cell, and other histologies, as well as International Federation of Gynecology and Obstetrics stages I to IV tumors. The control population was composed of healthy women free of gynecologic abnormalities (healthy controls), women undergoing surgery for benign ovarian conditions (benign controls), and women undergoing gynecologic surgery who were free of any ovarian disease (surgical controls). The composition of the sample is summarized in Table 1. Sera from cases, benign controls, and surgical controls were collected at the clinical visit before surgery, if possible, or in the operating room before surgical removal of the ovaries and before any treatment. Sera from healthy controls were obtained from women participating in a screening trial (20, 21) or a routine mammography screening (DAM 17-02-1-0691) and for women at average or intermediate risk for ovarian cancer. Controls were distribution-matched to cases based on age. The composition of the subset used to compare Bp/bAb assays to mAb assays (n = 214) is similar in terms of case status, histologic groups and stages of disease to the full set (Table 1).

Blood samples for all cases and controls were collected in serum separator tubes (BD Vacutainer, Becton, Dickinson and Company) and were processed according to the manufacturer's instructions. Blood was allowed to coagulate at room temperature for at least 30 min but no longer than 4 h. The serum was aliquoted and stored at -80°C until analysis (3-5 y). All specimens were coded with a unique vial identification number to assure that all assays would be done blinded to case status.

Bead-based immunoassays. The mesothelin mAb bead–based assay was done similarly to the HE4 mAb assay that was described previously (3, 22). Briefly, assays were done using filter plates (Millipore Corporation) with a vacuum manifold (Millipore) to remove assay reagents and wash the coupled microspheres. All incubations were carried out at room temperature, in the dark, with gentle agitation. Capture mAb (anti-mesothelin 4H3) was covalently coupled to carboxy-coated microspheres with the following modified buffers: the first bead activation buffer (AB1) was made with 0.1 mol/L sodium phosphate (NaH_2PO_4; pH 6.2; Sigma) and the second (AB2) with 1-ethyl-3-[3dimethylaminopropyl] carbodiimide hydrochloride (Pierce) and *N*-hydroxysulfosuccinimide (S-NHS; Pierce) diluted respectively to 38 and 109 mg/mL in AB1. The coupling buffer was made with 0.05 mol/L MES (pH 5.0; Sigma-Aldrich). Washes were done with PBST; assays, bead blocking and storage were done in PBS supplemented with 1% PBS (1% bovine serum albumin; Sigma-Aldrich). All assays of patient samples were run using the same lot of coupled microspheres and of biotin-conjugated antibody ovcar569 mAb detected with streptavidin-phycoerythrin.

Mesothelin and HE4 Bb/pAb bead–based assays were done as described above with the following differences: 50 μg/mL of anti-HE4 (18) or anti-mesothelin (R&D Systems) pAbs were covalently coupled to the carboxy-coated microspheres and used to capture recombinant proteins spiked in buffer or in control-pool serum (NHS). Captured proteins were detected by 5 μg/mL of anti-HE4 Bbs or 1 μg/mL of anti-mesothelin Bbs preincubated with PJ31S diluted 1,000-fold for HE4 or 2,000-fold for mesothelin in PBS (1% bovine serum albumin) on ice in the dark for 30 min. Bbs preincubated with PJ31S were added to microspheres preincubated with diluted sera and incubated for 30 min. Plates were analyzed with the Bio-Plex Array Reader.

Statistical analyses. The precision of Bb/pAb assays was assessed by calculating the coefficients of variation (CV) among the pooled serum

replicates within each run (plate) and across all runs using the *R* statistical programming language (version 2.3.1, R Development Core Team). CVs were calculated and reported on the raw scale to ensure comparability with other standard assays. For all other analyses, the serum levels were transformed from the raw scale as follows: after a log transformation, all markers were transformed by centering and scaling observations so that healthy controls have mean of 0 and variance of 1. This standardized scale promotes comparison between two markers because their scales are the same. These transformations leave receiver-operator characteristic (ROC) curves and their *P* values unchanged (23). The STATA statistical software package (version 9.0, Stata Corporation) was used for these analyses.

The equivalence of the Bb/pAb and mAb assays was assessed in a subset (n = 214) of the full serum set by calculating the Pearson correlation coefficient (24) between the serum concentration in the mAb assay and the Bb/pAb assay. For each marker, equivalence was assessed both overall and within subgroups defined by case status, within cases by stage and histologic group, and within controls by source.

The diagnostic accuracy of the Bb/pAb assays was assessed by estimating the ROC curves and area under the curve (AUC) statistics (25) for cases versus all controls, cases versus healthy controls, and cases versus benign surgical controls in the full serum set (n = 336). An AUC value of 1.0 represents perfect performance of the marker and 0.50 indicates a level of performance that is expected by chance alone. We also compared the classification performance and equivalency of the mAb and Bb/pAb assays for each marker in the serum subset (n = 214) using the nonparametric methods developed by DeLong et al. (26).

As serous carcinoma is the most common and the most lethal type of ovarian cancer, it is of particular interest for early detection research. A composite marker (CM) between the HE4 and mesothelin Bb/pAb assays was defined using weighed linear combinations of the standardized markers in the full serum set excluding the nonserous cases for serous cases versus controls (n = 293). Logistic regression was used to estimate the weights for the combination of the two markers and to test whether or not the CM improves prediction over either marker alone (27). We then compared the ROC curves for the CM to that for each individual marker.

Results

Development and optimization of Bb/pAb assays. Anti-HE4 Bbs (8) and anti-mesothelin Bbs (9) were tested for their ability to complement antigen-specific pAbs in ELISA assays done on fluorescent microspheres (bead-based assays; Fig. 1). Assays were first calibrated with HE4 or mesothelin recombinant antigens secreted as fusion protein by mammalian cells (meso-Ig; ref. 22) or yeast (HE4y and meso-7; ref. 9). Diploid secreted, *in vivo* b-HE4y was validated by detection ELISA. Figure 2A shows that b-HE4y secreted by yeast clones 9 and 17 were strongly detected with two anti-HE4 mAbs (3D8 or 2H5). b-HE4y secreted by yeast clone 17 was used for the rest of the study.

We compared the ability of two anti-mesothelin Bbs P2 and P4 (9) to detect meso-Ig in a double-determinant ELISA format

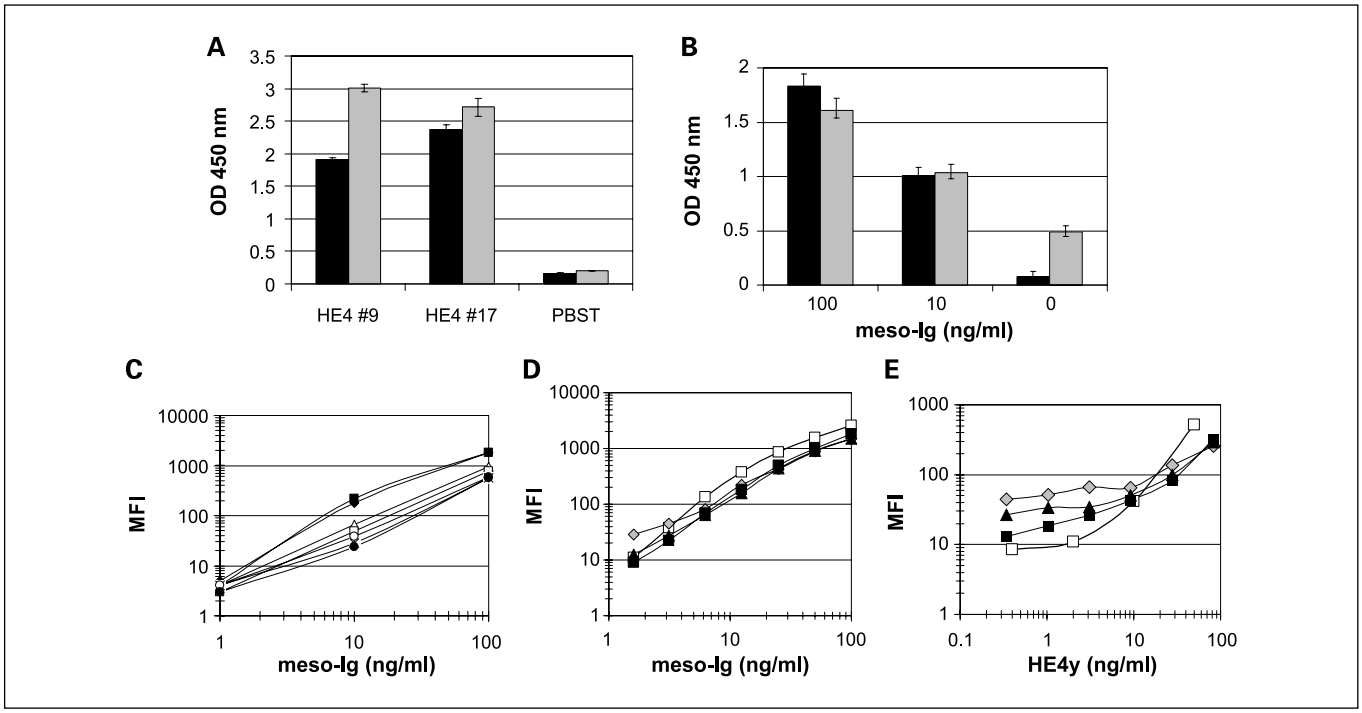

Fig. 2. Development of Bb/pAb assays. *A,* validation of diploid-secreted b-HE4y recombinant protein. Ni-purified b-HE4y was immobilized on a streptavidin plate and detected with 3D8 mAb (*black columns*) or 2H5 mAb (*gray columns*) followed by horseradish peroxidase – conjugated antimouse immunoglobulin. As a negative control, wells were coated with PBST only. *B,* comparison of ability of anti-mesothelin Bbs to capture a mesothelin recombinant protein (meso-Ig). Anti-mesothelin P4 (*black columns*) and P2 (*gray columns*) Bbs were immobilized on a streptavidin plate and incubated with serial dilutions of meso-Ig. Captured recombinant proteins were detected with horseradish peroxidase – conjugated antihuman immunoglobulin. *C,* optimization of anti-mesothelin Bb/pAb assay. Anti-mesothelin pAb-coated fluorescent microspheres were incubated with serial dilutions of meso-7 in buffer. Captured proteins were detected with P4 Bb premixed with seven different types of modified fluorescent Streptavidin R-PE: black diamonds, PJ31S; black squares, PJLS; white triangles, PJ35S; white squares, PJ37S; stars, PJ39S; white circles, PJ33S; black circles, streptavidin-phycoerythrin. *D* and *E,* detection ranges of anti-mesothelin and HE4 Bb/pAb assays: anti-mesothelin (*D*) or anti-HE4 (*E*) pAb-coated fluorescent microspheres were incubated with serial dilutions of meso-Ig (*D*) or HE4y (*E*) recombinant proteins diluted in buffer (*white squares*) or in serial dilutions of NHS (20-fold, *gray diamonds*; 10-fold, *black triangles*; 5-fold, *black squares*). Captured proteins were detected with P4 Bb (*D*) or anti-HE4 Bb pool (*E*) premixed with PJ31S streptavidin.

Table 1. Characteristics of the women in the serum sets

Characteristics	Serum subset, *n* = 214	Full serum set, *n* = 336
Age, median (range)	57.0 (25.0 – 83.0)	57.0 (19.0 – 87.0)
Case status, *n* (%)		
Healthy controls	58 (27.10)	94 (27.98)
Benign controls	53 (24.77)	81 (24.11)
Surgical controls	32 (14.95)	45 (13.39)
Cases	71 (33.18)	116 (34.52)
Stage, *n* (%) for cases only		
Stage I	23 (32.40)	33 (28.45)
Stage II	4 (5.63)	9 (7.76)
Stage III	43 (60.56)	72 (62.07)
Stage IV	1 (1.41)	1 (0.86)
Unstaged	0	1 (0.86)
Histology, *n* (%) for cases only		
Serous	44 (61.97)	73 (62.93)
Mucinous	6 (8.45)	9 (7.76)
Endometrioid	6 (8.45)	10 (8.62)
Clear Cell	5 (7.04)	11 (9.48)
Other	10 (14.09)	13 (11.21)

NOTE: Samples were obtained through the POCRC and were collected before surgical removal of the ovaries. Cases were defined as having invasive epithelial carcinoma confirmed by standardized review of medical records and pathologist examination of paraffin-embedded tissue. Histologies represented in each set included serous, mucinous, endometrioid, clear cell, and others. International Federation of Gynecology and Obstetrics stages I to IV tumors were also included in each serum set.
The serum subset contains all of the samples included within the full serum set.

(Fig. 2B). P2 and P4 Bbs specificity for mesothelin was detailed in a previous publication (9). Both Bbs were able to quantify meso-Ig, but the background generated by P2 Bbs was much higher than that of P4 (Fig. 2B); thus, P4 Bbs were selected for use in the rest of the study. In addition, the signal intensity could be increased 10-fold using the modified streptavidin conjugates PhycoLink Streptavidin R-PE PJ31S or PJLS (Fig. 2C). Finally, anti-mesothelin Bbs could also accurately quantify meso-Ig spiked in serial dilutions of buffer or NHS (Fig. 2D). The sensitivity of the assay was in the nanogram-per-milliliter range at all tested dilutions of meso-Ig recombinant protein. But when the test was used to measure native serum mesothelin, the greatest difference between case and control serum pools was observed with a serum dilution of 5-fold (data not shown). Thus serum dilution 5 was chosen for the rest of the study.

The HE4 Bb/pAb assay was developed similarly to the mesothelin Bb/pAb assay. Anti-HE4 Bb pool (8) was premixed with Streptavidin R-PE PJ31S, and the assay was calibrated using HE4y recombinant protein (Fig. 2E). Anti-HE4 pAb was immobilized on carboxy-coated microspheres. Dilution of HE4y protein in serial dilution of NHS changed the slope of the curves compared with the signal generated by HE4y protein diluted in buffer (Fig. 2E), but the overall assay sensitivity remained in the nanogram-per-milliliter range. Serum dilution 10 was chosen for the rest of the study.

Reproducibility of HE4 and mesothelin Bb/pAb assays. Results of the reproducibility experiments are reported in Table 2. Across all dilution levels, the average within-run and total (across runs) CVs for the mesothelin Bb/pAb assay were 14.0 and 14.5, respectively. The average within-run and total CVs for the HE4 Bb/pAb assay were 11.7 and 23.8, respectively. The CVs showed a moderate trend by dilution level, being somewhat higher for serum pools from healthy women than from cases.

Validity of HE4 and mesothelin Bb/pAb relative to mAb assays. We used the serum subset (n = 214) to directly compare the HE4 Bb/pAb assay to a previously developed HE4 bead–based assay using mAbs (3) and the mesothelin Bb/pAb assay to a mesothelin bead-based assay developed with 4H3 and ovcar 569 mAbs (12). As reported in Table 3, the mesothelin and HE4 Bb/pAb assays were both highly correlated with their mAb counterpart when all cases and controls were included in the analyses (0.90 and 0.89,

Table 2. CV by dilution level, within run and total

Dilution level	HE4		Mesothelin	
	Within run	Total	Within run	Total
Case-pool only	7.3	17.8	14.7	15.6
1:1 dilution pool	12.6	21.7	12.8	13.4
1:3 dilution pool	11.1	24.5	13.9	13.9
1:7 dilution pool	12.5	25.8	14.6	15.3
1:15 dilution pool	12.6	26.7	12.0	12.4
Healthy pool only	14.0	26.1	16.1	16.1
Across levels	11.7	23.8	14.0	14.5

NOTE: Assay precision was assessed in reproducibility experiments using pooled sera from ovarian cases and controls obtained from the POCRC repository. Dilution levels represent case-pool serum (formed by combining 1 mL of serum from each of 50 patients diagnosed with late stage ovarian cancer), control-pool serum (made from serum collected from seven healthy female volunteers), and intermediate pools of serum (created by serial dilution of the case pool with control pool by factors of 2). The reproducibility experiment was conducted for each analyte using 16 replicates of each serum dilution in each of four runs (plates), providing a total of 64 replicates for each analyte level. The column titled "within run" represents the average CVs within each plate. The column titled "total" represents the CVs across all plates.

Table 3. Correlation between the Bb/pAb and mAb assays by markers and characteristics of the women in the serum subset (n = 214)

Characteristics	*n*	Mesothelin bead-based assays		HE4 bead-based assays	
		r	*P*	*r*	*P*
All cases and controls	214	0.8993	<0.001	0.8920	<0.001
Cases only	71	0.9192	<0.001	0.9504	<0.001
Early stage (stage I)	23	0.9147	<0.001	0.7885	<0.001
Stages II-IV	48	0.8955	<0.001	0.9493	<0.001
Healthy controls	58	0.7440	<0.001	0.1508	0.2629
Benign controls	53	0.7225	<0.001	0.4161	0.002
Surgical controls	32	0.7943	<0.001	0.5293	0.002
Serous	44	0.8945	<0.001	0.9536	<0.001
Mucinous	6	0.8237	0.044	0.4914	0.3222
Endometrioid	6	0.8634	0.027	0.9905	<0.001
Clear cell	5	0.9367	0.019	0.7118	0.178
Other histologies	10	0.9078	<0.001	0.9548	<0.001

NOTE: Pearson's correlation coefficients between the mAb and Bb/pAb assays for each marker by case status, stage, and histology. The correlation coefficient quantifies the strength of the relationship between the mAb and Bb/pAb assays, where 1.0 indicates a perfect correlation between the assays.

respectively). This correlation was strengthened to 0.92 for mesothelin and 0.95 for HE4 when cases alone were evaluated. The correlation remained strong for mesothelin assays regardless of the histologic group or stage of disease under evaluation (Table 3). In addition, there was a strong correlation between the HE4 Bb/pAb and HE4 mAb assays among early-stage cases (0.78), late-stage cases (0.95), and women with serous (0.95) or endometrioid histologies (0.99). The correlation between the two HE4 assays among the healthy, benign, and surgical controls (Table 3) was relatively low, due in part to the very low values and variability of HE4 in these categories of samples.

Diagnostic accuracy of HE4 and mesothelin Bb/pAb assays. We also compared the classification performance of the mAb and

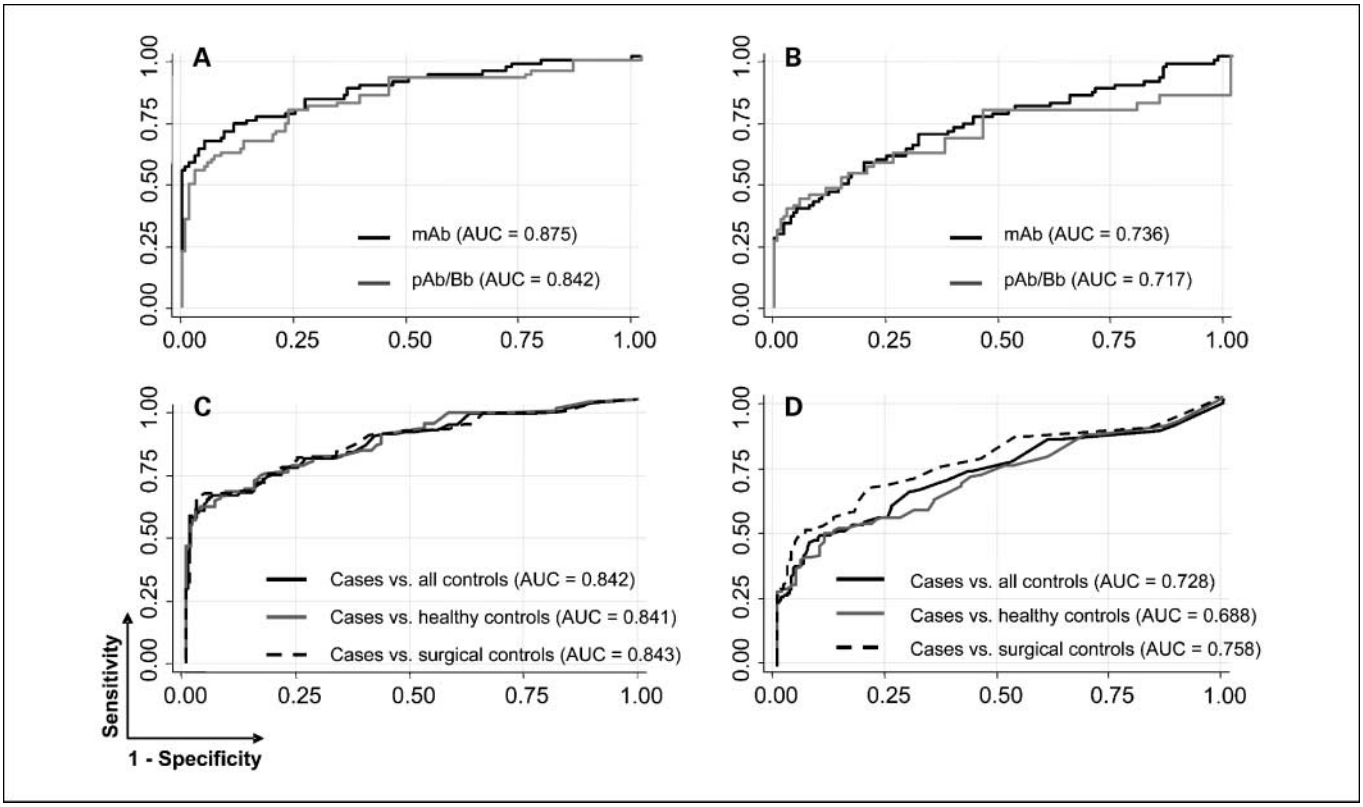

Fig. 3. Comparison of diagnostic performance of Bb/pAb and mAb bead-based assays in the serum subset (n = 214) and in the full serum set (n = 336) for cases versus controls, cases versus healthy controls, and cases versus surgical controls. *A,* ROCs for HE4. *B,* ROC's for mesothelin. *C,* ROCs for HE4 Bb/pAb assay. *D,* ROCs for mesothelin Bb/pAb assay.

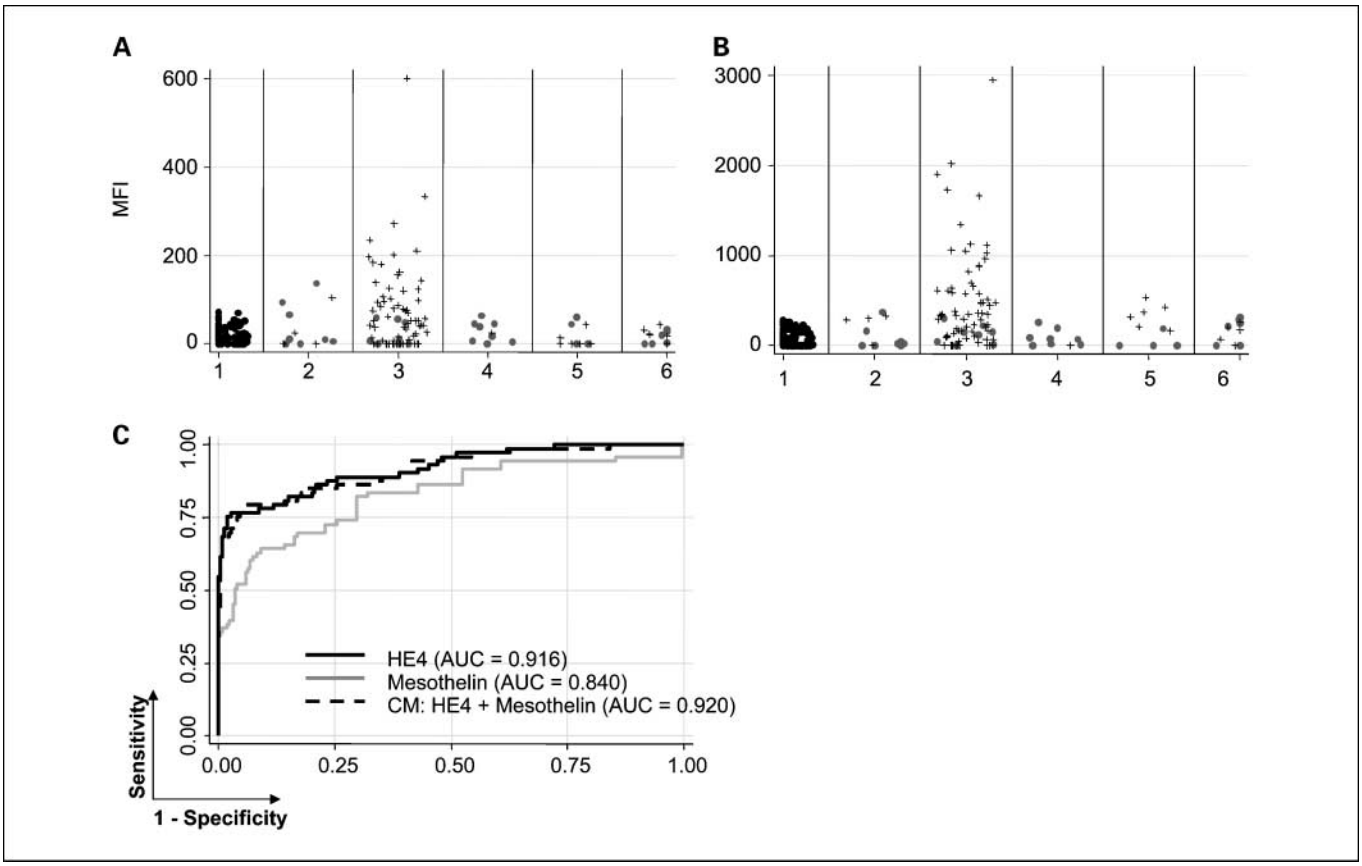

Fig. 4. Diagnostic performance of HE4 and mesothelin Bb/pAb assays in the full serum set (n = 336). *A* and *B,* diagnostic performance by histology. Median fluorescence intensity (*MFI*) values of mesothelin (*A*) and HE4 (*B*) as measured by Bb/pAb assays; controls (*1*) and 2 to 6 ovarian cancers (gray circles, early stages; crosses, late stages): clear cell (*2*), serous (*3*), mucinous (*4*), endometrioid (*5*), and others (*6*). *C,* CM = 1.12(HE4) + 0.45(mesothelin). Weight for HE4 = 0.71. Weight for mesothelin = 0.29.

Bb/pAb assays for each marker in the serum subset (n = 214). The AUCs for the mesothelin mAb and Bb/pAb assays were not significantly different (0.74 and 0.72, respectively; ref. 26; P = 0.38). For HE4, the corresponding AUCs (0.88 and 0.84, respectively) were slightly higher than for mesothelin and not significantly different (P = 0.20; Fig. 3A and B).

We evaluated the analytic sensitivity of the HE4 and mesothelin Bb/pAb assays at 95% and 98% specificity in the expanded serum set (n = 336). At 95% and 98% specificity, the HE4 Bb/pAb assay obtained sensitivity levels of 62.07% and 55.17%, respectively. The corresponding sensitivity levels for the mesothelin Bb/pAb assay were 37.93% and 26.72%. The performance and diagnostic accuracy of the Bb/pAb assays to discern cases from all controls, cases from healthy controls, and cases from surgical controls was also evaluated in the expanded serum set (n = 336). The AUCs for the HE4 Bb/pAb assay in differentiating cases from all controls, cases from healthy controls, and cases from surgical controls were indistinguishable from each other (0.84; Fig. 3C). The diagnostic performance of the mesothelin Bb/pAb assay was not quite as good, with AUC values of 0.73, 0.69, and 0.76 respectively (Fig. 3D).

Compared with controls, HE4 and mesothelin serum titers were significantly elevated in serous but not among other histologic subtypes (Kruskil-Wallis test, $P < 0.001$ for HE4 and $P < 0.001$ for mesothelin; Fig. 4 A and B). Because serous cancer is of particular interest with respect to early detection, the performance of HE4, mesothelin, and their CM was evaluated for serous cases versus controls. The CM was defined using a weighed linear combination of the normalized marker values with the following formula: CM = 1.12(HE4) + 0.45(mesothelin). Although HE4 carried 71% of the weight in the CM, both markers were significant predictors in the logistic regression model (HE4 $P < 0.001$; mesothelin P = 0.013). As illustrated in Fig. 4C, the AUCs for HE4 and mesothelin alone were 0.92 and 0.84, respectively. The AUC for the CM was 0.92, with sensitivity of 77% at 95% specificity and 75% at 98% specificity.

Discussion

New proteomics technologies are producing hundreds of candidate biomarkers for potential use in diagnosis, early detection, and risk assessment of cancer. Evaluation and validation of these candidates are challenged by the need for high-quality annotated human samples, particularly "preclinical" blood samples collected several months to several years before the diagnosis of cancer, and especially by the need for high-affinity reagents for use in the development of immunoassays. We sought to develop a strategy for rapid development of specimen-efficient assays when complementary mAbs are not commercially available for use in a sandwich assay. For novel markers for which no mAbs are available, it will not be

possible to compare a novel assay to a gold standard. We therefore sought to show the validity of the assay method using two serum markers for which mAbs are available.

We found the Bb/pAb assays to be comparable in their precision and performance to research-quality assays using mAbs in our laboratory. The total CV for the HE4 Bb/pAb assay ranged from 17.8 for the case pool sera to 26.1 for the healthy pool sera, which compares favorably to the same estimates for the HE4 mAb bead-based assay which ranged from 26.6 for case pool sera to 32.6 for the healthy pool sera (3). The average total CV for the mesothelin Bb/pAb assay was 14.5, which is acceptable for a research assay. We found that the Bb/pAb and mAb assay results correlated highly and the diagnostic performance or ability to discriminate between cases and controls did not differ between the Bb/pAb and mAb assays, suggesting that the former are appropriate for use in validation studies. Material required for Bb/pAb assays was even less than the 15 μL of serum needed for the mAb bead-based assays: the mesothelin Bb/pAb requires 12 μL per sample and the HE4 Bb/pAb requires 6 μL per sample.

Preclinical samples from large studies, such as the Women's Health Initiative and the Prostate, Lung, Colon and Ovary trial are accessible for serum marker validation studies, but the quantity of material available for each participant is very limited. Standard ELISAs using mAbs often require 50 to 200 μL of serum. Moreover, development of assays that depend on mAbs produced in mice is time-consuming and expensive, and identification of compatible pairs of mAbs for sandwich ELISAs can be challenging. To accelerate the assay development process, we used a new class of recombinant antibodies (Bbs) in combination with available pAbs (Bb/pAb assays) to form sandwich assays that can be run as bead-based assays. The procedure to obtain antigen-specific Bbs is remarkably short compared with the classic methods of antibody production. The first step, identification of a pool of antigen-specific yeast-display scFv from a naive library (19) done by magnetic and flow sortings, takes 2 to 3 weeks. Conversion of the scFv from yeast-display to yeast-secreted Bbs is next achieved within 2 weeks by a PCR amplification of the scFv-encoding cDNA from the whole-yeast DNA, followed by a cloning by gap repair in the vector pTOR2 and mating of the yeast secreting scFv with BIRA-transformed yeast. Resulting diploids secrete Bbs, whose specificity and sensitivity can be determined using classic immunologic methods, such as Biacore, flow cytometry, and ELISA assays (8).

Bead-based assays require smaller amounts of serum and are efficient for high throughput screening, whereas isolation of Bbs from yeast is rapid, easy, and relatively inexpensive. This novel approach enabled us to develop sensitive and reliable assays for use in large population validation studies. Bb/pAb assays for the detection of serum HE4 and mesothelin in ovarian carcinoma patients did well in 336 samples collected through the POCRC, yielding performance comparable with that of mAb bead-based assays.

We used our novel assays to evaluate the performance of the two markers alone and in combination in serous ovarian cancer. Our results suggest that HE4 and mesothelin are better serum markers of serous than of endometrioid, clear cell, or mucinous ovarian cancers. Serous cancer is the most common form of ovarian cancer and the least likely to be diagnosed, while, it is still confined to the ovary. Recent reports suggest that at least in BRCA1 mutation carriers, serous pelvic cancer may arise from dysplasia and/or early malignancy in the fimbrial end of the fallopian tube (28), metastasizing to other epithelial cells, including those on the surface of the ovary as well as those that line the peritoneum. HE4 is expressed by epithelial cells in the normal fallopian tube but not by epithelial cells of the normal ovary; interestingly, cells lining inclusion cysts of the ovary do express HE4, and both serous and endometrioid epithelial ovarian cancers overexpress HE4 (18). Mesothelin is a GPI-anchored protein constitutively expressed by the epithelial cells on the peritoneal wall and by cancer cells from diverse origins, including ovarian, pancreatic, and mesothelioma (10). However, despite its normal expression by peritoneal cells, soluble mesothelin is found only in ovarian cancer sera and ascites and in mesothelioma sera and pleural effusions (12, 16, 17). Altogether, this strongly suggests that the cell surface expression or overexpression of a protein cannot be simply correlated to its specific presence in patient fluids. Multiple other factors, such as a higher level of GPI-PLD enzyme activity (29), may contribute to the production of serum biomarkers and underlines the critical need for high-quality tools for serum marker validation studies. The novel approach using Bbs described in this manuscript enables the development of sensitive and reliable, yet cost– and time-effective, assays for use in large population validation studies.

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