ELSEVIER

CHARACTERIZATION OF THE BRCA1-LIKE IMMUNOREACTIVITY OF HUMAN SEMINAL PLASMA

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ABSTRACT

Objectives. The subcellular localization of the breast cancer susceptibility gene product BRCA1 has been controversial. Discrepant results have been reported during the past 3 years, partially because of the unavailability of highly specific reagents for BRCA1 protein. Our objective was to characterize the BRCA1-like immunoreactivity that is detected in human seminal plasma by using monoclonal and polyclonal antibodies that are supposedly specific for BRCA1 protein.

Methods. We used immunologic, chromatographic, and protein sequencing techniques to detect the immunoreactivity of BRCA1 in seminal plasma and to purify and partially identify the immunoreactive species.

Results. We present data indicating that two BRCA1 antibodies, SG-11 and D-20, which were thought to be free of cross-reactivities, strongly interact with proteins present in human seminal plasma. This cross-reactivity is detectable even at seminal plasma dilutions as high as 10^6-fold, and it is effectively blocked by peptides that capture the binding site of either SG-11 or D-20 antibodies. Purification and characterization of the immunoreactive compound revealed that this consists of a macromolecular complex that contains semenogelins. The D-20 polyclonal antibody was found to cross-react with purified semenogelins I and II; the SG-11 monoclonal antibody appeared to recognize a component of the macromolecular complex that was not semenogelin.

Conclusions. Our data demonstrate that the BRCA1 antibodies SG-11 and D-20 strongly interact with seminal plasma proteins and are not highly specific for BRCA1 protein. It is thus suggested that BRCA1 antibodies should be used with caution until reagents free of interference are developed and evaluated. In light of the very high cross-reactivity of the two antibodies with seminal plasma proteins, we recommend that new BRCA1 antibodies should be examined for cross-reactivity with seminal plasma proteins to verify specificity.

UROLOGY **54:** 753–762, 1999.

The breast and ovarian cancer susceptibility gene BRCA1 is mutated in the germline, and the normal allele is lost in tumor tissue from hereditary breast and ovarian cancer.[1,2] Mutations in the BRCA1 gene are thought to account for approximately 45% of families with a significantly high incidence of breast cancer and at least 80% of families with an increased incidence of early onset breast and early onset ovarian cancer.[1] Transfection of the wild-type BRCA1 gene has been shown to inhibit growth of breast and ovarian cancer cells but not of other malignant cells.[3] This observation suggested that BRCA1 acts as a tumor suppressor, but the mechanism through which this molecule confers its growth inhibitory function remains obscure.

BRCA1 protein consists of 1863 amino acids and contains a highly conserved N-terminal RING finger domain similar to that found in other proteins interacting with nucleic acids or forming protein complexes.[4] The RING finger motif and two putative nuclear localization signals in BRCA1 are compatible with reports on its nuclear localization.[5–7]

Diagnostic Systems Laboratories provided financial support for this study.

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Submitted: November 13, 1998, accepted (with revisions): April 28, 1999

0090-4295/99/$20.00
PII S0090-4295(99)00233-2

Chen and colleagues[5] demonstrated that BRCA1 is present in the nucleus of normal cells but is aberrantly localized to the cytoplasm in breast and ovarian tumor cells. Scully *et al.*[6] suggested that the BRCA1 protein is exclusively present in the nucleus regardless of cell type, yet Jensen *et al.*[8] showed that BRCA1 is a membranous and secreted protein containing a consensus granin sequence. These different conclusions may be attributed to the fact that the three groups studied the BRCA1 subcellular localization using different antibodies and different experimental techniques. It has been shown that BRCA1 antibodies against its C-terminus are not highly specific for this protein and they exhibit cross-reactivity with the human epidermal growth factor receptor and other proteins.[9] The BRCA1 antibody most susceptible to cross-reactivity by non-BRCA1 proteins appears to be the C-20 antibody. This rabbit antibody recognizes amino acids 1843–1862 of the BRCA1 carboxylterminus. Scully *et al.*[6] developed a panel of monoclonal antibodies against BRCA1 to examine the localization of this protein in various cell types. These antibodies are now commercially available.

In an effort to investigate subcellular localization and tissue expression, our group has recently developed an immunoassay for the quantitative detection of this protein.[10] In that report, we showed that an abundant seminal plasma protein is recognized by BRCA1-specific antibodies and thus detected by our assay. In the present study, we undertook a strategy to purify and sequence this immunoreactive protein to identify whether it represented BRCA1, a BRCA1-homologue, or a non-homologous cross-reacting protein.

MATERIAL AND METHODS

BRCA1 Reagents

The following antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, Calif: polyclonal antibody C-20, generated by immunizing rabbits with peptide LYQCQELDTYLIPQIPHSHY (peptide C-20) and polyclonal antibody D-20, generated by immunizing rabbits with peptide DLSALRVEEVQNVINAMQKI (peptide D-20). Peptide C-20 maps to the C-terminal end of human BRCA1 (amino acids 1843–1862) and peptide D-20 to the aminoterminus (amino acids 2–21). The immunizing peptides were also purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody SG-11 was raised against a peptide representing amino acids 1846–1863 and recognizes the carboxylterminus of human BRCA1. SG-11 was purchased as a 0.1 mg/mL solution in gelatin (Calbiochem, San Diego, Calif) or as a 4 mg/mL solution in a phosphate buffer without any other additives (custom produced by Calbiochem). The irrelevant mouse monoclonal antibodies antisomatotropin (antigrowth hormone), antilutropin, antiamylase, and anticarcinoembryonic antigen were purchased as 1 mg/mL preparations (Medix Biochemica, Kauniainen, Finland) and the irrelevant peptide YRSPHWGSTYSV was purchased from Research Genetics, Huntsville, Ala.

Semenogelin Reagents

A mouse monoclonal antibody against semenogelins I and II, coded M9, was used in this study. This antibody has been previously described.[11] Full-length semenogelins I and II were purified from seminal plasma using previously established procedures.[12] During these experiments, we also used a polyclonal antibody raised by immunizing rabbits with human seminal plasma; we refer to this reagent as antiserum B. This antibody is not specific for any particular seminal plasma protein.

Seminal Plasmas

Seminal plasmas were obtained by centrifugation of human semen samples submitted for fertility studies. These samples were kept at −20°C until analysis. We used a number of different seminal plasmas for the studies described here.

Tissue Extracts

Tissue specimens, obtained during autopsy, were stored at −80°C until analysis (less than 1 week). About 200 mg of tissue was pulverized to a fine powder, and the cells were lysed for 30 minutes on ice with 2 mL of lysis buffer (50 mmol/L Tris buffer, pH 8.0, containing 150 mmol/L sodium chloride, 5 mmol/L ethylenediaminetetraacetic acid, 10 mL/L NP-40 surfactant, and 1 mmol/L phenylmethylsulfonyl fluoride). The lysate was centrifuged at 14,000*g* at 4°C for 30 minutes, after which the supernatant was collected and used for analysis.

Antibody Biotinylation

The biotinylation reagent NHS-LC-LC-Biotin (Pierce Chemical, Rockford, Ill) was used as described elsewhere.[13] The biotinylated antibodies were dialyzed twice against 5 L of a 0.1 M bicarbonate solution to remove excess biotin.

Immunoassays

Antibodies and Solutions. The goat antimouse immunoglobulin G (GAMIg) antibody and the goat antirabbit IgG antibody conjugated to alkaline phosphatase (GARIg-ALP) were supplied as 1 mg/mL preparations (Jackson Immunoresearch, West Grove, Pa). Dilution of the microtiter well-coating GAMIg antibody was made in 50 mmol/L Tris, pH 7.4, containing 7.5 mmol/L NaN_3. Buffer A was a 50 mmol/L Tris buffer, pH 7.8, containing 60 g/L bovine serum albumin, and 7.5 mmol/L NaN_3. Buffer B was the buffer A solution with 0.5 mol/L KCl, 5 g/L Tween 20, 0.5 g/L mouse IgG, and 100 mL/L goat serum. Buffer C consisted of buffer A with 0.5 mol/L KCl and 0.2 g/L goat IgG.

Procedure. All assays were based on the general configuration shown in Figure 1. Ninety-six-well, white polystyrene plates were incubated overnight at 4°C with 100 μL/well of GAMIg antibody diluted 500-fold in coating buffer. After six washing cycles, the wells were incubated with 100 μL of SG-11 antibody (or M9 or irrelevant antibodies) diluted 500-fold in buffer A for 2 to 6 hours at room temperature with shaking. All subsequent steps were performed at room temperature with shaking. After another six washing cycles, seminal plasmas or high-performance liquid chromotographic (HPLC) fractions were diluted from 10- to 10^6-fold in buffer A and added in 100-μL volumes to the wells for 1 hour of incubation. The plates were again washed as above and 100 μL of D-20 antibody diluted 1000-fold in buffer B (or antiserum B diluted 10,000-fold in buffer B) were added for another 1-hour incubation. Six cycles of washing were followed by a 1-hour incubation with 100 μL/well of GARIg-ALP diluted 2000-fold in buffer C. Detection of alkaline phosphatase activity was accomplished using time-resolved fluorometry, as described elsewhere.[14]

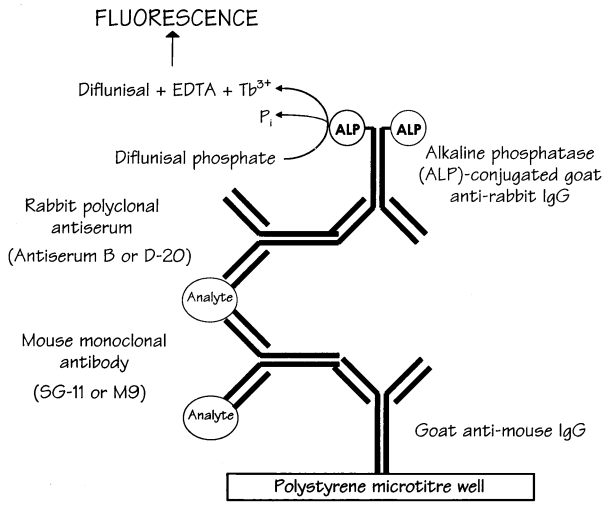

FIGURE 1. *General immunoassay configuration. Goat antimouse immunoglobulin is coated onto polystyrene microtiter wells. The capture mouse monoclonal antibody (SG-11, M9, or other irrelevant antibody) binds the analyte of interest, which is subsequently recognized by a rabbit polyclonal antibody (D-20 or antiserum B). Antibody binding is detected by an alkaline phosphatase-conjugated goat antirabbit immunoglobulin and time-resolved fluorometry. P_i = inorganic phosphate.*

Standardization. We selected one seminal plasma that exhibited very high immunoreactivity when analyzed with the assay operating with the SG-11 antibody as the capture antibody and either antiserum B or D-20 antibody as the detection antibodies. We arbitrarily assigned a concentration of this seminal plasma as being 10^6 U/L and prepared standards of various concentrations from 0.1 to 5000 U/L, the latter concentration corresponding to a 2000-fold dilution of the seminal plasma in buffer A.

Chromatographic Studies

We used a Hewlett Packard 1100 HPLC system. The procedures are detailed in the following sections.

Gel Filtration Chromatography. Two different columns were used: Superdex 200 FPLC (Pharmacia Biotech, Montreal, Canada) and a TSK-Gel G3000SW column, 600 × 7.5 mm (Tosoh-Haas, Montgomeryville, Pa). The first column was run isocratically at 0.4 mL/min with a mobile phase of 0.1 mol/L phosphate buffer, pH 7.1, containing 0.15 mol/L of NaCl. The second column was run with a mobile phase of 0.1 mol/L sodium phosphate and 0.1 mol/L sodium sulfate, pH 6.80, at a flow rate of 0.5 mL/min. Fractions of 0.4 mL or 0.5 mL were collected for analysis. Both columns were calibrated with a molecular weight standard solution from BioRad (Richmond, Calif). Data obtained using the two columns were similar.

Ion-Exchange Chromatography. An HR 10/16 column was packed with CM-Sepharose Fast Flow packing material (Pharmacia Biotech) and used for ion-exchange chromatography. Buffer A was a 0.05 mol/L CH_3COONa solution, pH 6.0, and buffer B was a 0.05 mol/L CH_3COONa/1 M NaCl solution, pH 6.0. Seminal plasma (500 μL) was mixed with buffer A (1500 μL) and manually injected onto the column. During the first 20 minutes of the run, the flow rate was 1 mL/min with buffer A, and it was subsequently increased to 3 mL/min. The linear gradient was then run for 40 minutes (0% to 100% buffer B). Protein fractions were collected every minute.

Polyacrylamide Gel Electrophoresis and Immunoblotting

CM-Sepharose fractions were concentrated approximately 100 times with Centriprep-10 concentrators (Amicon, Beverly, Mass). These fractions or nonconcentrated fractions from the gel filtration columns were mixed with three volumes of sample buffer (Novex, San Diego, Calif) containing 10% dithiothreitol. After denaturation at 70°C for 10 minutes, the samples were applied onto one-well 4% to 12% minipolyacrylamide gels (Novex) and electrophoresed at 200 V for 35 minutes. After electrophoresis, the separated proteins were transferred to nitrocellulose membranes (or a polyvinyldifluoride [PVDF] membrane for protein sequencing) by electroblotting at 25 V for 1 hour. The membranes were then treated overnight in blocking solution (5% nonfat dried milk in wash solution; Tris-buffered saline, pH 7.6, 0.1% Tween 20), and subsequently probed for 1 hour with the antibody of interest diluted 200-fold in wash solution. Blocking experiments were performed by incubation of the probing antibody (SG-11 or D-20 or M9) for 1 hour with 100 μL of peptide C-20 or D-20. The peptide was added at a 100-fold molar excess over the amount of antibody. After washing, each blot was incubated for 1 hour with an antimouse or antirabbit antibody conjugated to horseradish peroxidase. After a final washing, antibody binding was visualized by chemiluminescence, captured on x-ray film, using the ECL-Western detection kit from Amersham.

Protein Sequencing

PVDF membranes obtained as described above were stained with Commassie blue and the bands of interest were cut. Proteins were eluted from these membrane strips and subjected to automated amino acid sequencing with the Edman degradation method.

RESULTS

Assay Specificity

A preliminary study by our group demonstrated that an abundant seminal plasma protein shares immunoreactive epitopes with BRCA1.[10] To examine the specificity of the assay used (Fig. 1), we carried out the following experiments.

The immunoassay using SG-11 as the capture antibody (C-terminal specific) and D-20 as the detection antibody (N-terminal specific) was performed at three concentrations of seminal plasma immunoreactivity: 10, 50, and 200 U/L (Fig. 2A). Incubation of SG-11 antibody with its specific peptide C-20 before the addition of the diluted seminal plasma resulted in a substantial reduction of the fluorescence signal; incubation of SG-11 with peptide D-20 (which does not bind to SG-11) or with another irrelevant peptide had no effect on the signal. Similarly, substitution of SG-11 by four other monoclonal antibodies (described in the Material and Methods section) did not yield any signal. These data demonstrate that the SG-11 antibody is necessary for signal generation, and that SG-11 captures this seminal plasma component through its binding site (defined by peptide C-20) and not through nonspecific interactions.

In the same assay configuration, substitution of

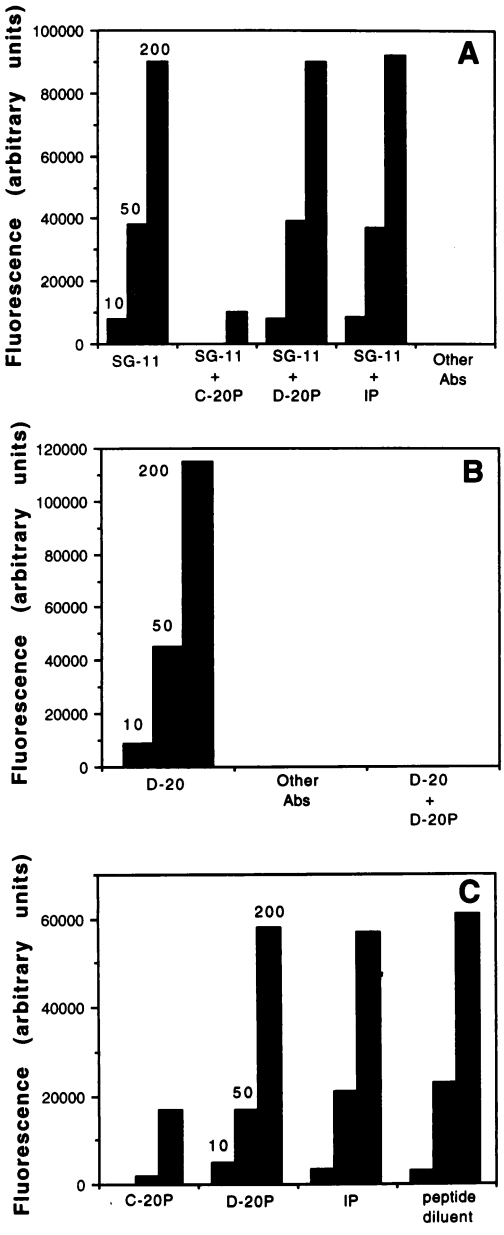

FIGURE 2. *Specificity of BRCA1 antibodies for the seminal plasma immunoreactive substance. Three arbitrary concentrations (10, 50, and 200 U/L) of the immunoreactive substance were used. (A) The assay was performed using as the capture antibody SG-11, SG-11 after incubation with peptide C-20 (SG-11 + C-20P), with peptide D-20 (SG-11 + D-20P), with an irrelevant peptide (SG-11 + IP), or after substituting SG-11 with irrelevant monoclonal mouse antibodies (other Abs). (B) The assay was performed using as the detection antibody D-20, irrelevant rabbit IgG (other Abs), or D-20 previously blocked with peptide D-20 (D-20 + D-20P). (C) Before the addition of the D-20 detection antibody, the GAMIg/SG-11/analyte complex was incubated with peptide C-20 or D-20, with an irrelevant peptide (IP) or with peptide diluent.*

the D-20 antibody by other rabbit IgGs (other antibodies) produced no signal (Fig. 2B). Similarly, no signal was generated when antibody D-20 was incubated with peptide D-20 before its addition to the assay (Fig. 2B). These data demonstrate that the immunoreactive substance captured by SG-11 specifically reacts with the D-20 antibody at the binding site defined by peptide D-20.

After incubation of seminal plasma with SG-11 capture antibody and before addition of the D-20 detection antibody, the GAMIg/SG-11/analyte complex was incubated with peptide C-20, with peptide D-20, with an irrelevant peptide, or with peptide diluent. As shown in Figure 2C, only C-20 peptide could effectively compete with and displace the captured immunoreactive substance from SG-11.

The described data demonstrate that there is a protein in seminal plasma that is specifically recognized, in a "sandwich-type" configuration, by the BRCA1-specific antibodies SG-11 and D-20. Separate experiments have shown that both SG-11 and D-20 antibodies can immunoprecipitate the immunoreactive proteins from seminal plasma (data not shown). Below, we describe further characterizations of this protein.

Tissue Expressing the Immunoreactive Protein

To identify the tissue that most likely produces and secretes this protein into the seminal plasma, we collected tissues from organs that contribute secretions into the semen. These included the testis, epididymis, vas deferens, seminal vesicle, and prostate. Spleen tissue was used as a negative control. Protein extracts were prepared from all tissue specimens, and the concentration of total protein in all extracts was adjusted to ~1 mg/mL. The extracts were then analyzed with the assay of Figure 1, using SG-11 as the capture antibody and D-20 as the detection antibody. As shown in Figure 3, the source of the BRCA1-like immunoreactivity in seminal plasma appears to be the seminal vesicles.

Characterization of the Immunoreactive Protein

Ion-exchange chromatography was performed on seminal plasma using the weak cation-exchange column CM-Sepharose. Under the conditions described in Material and Methods, most proteins (~90% of total protein) of seminal plasma elute at fractions 6–7 and 12–16 (data not shown). The immunoreactivity of all fractions was measured using the immunofluorometric assay with SG-11 as the capture antibody and antiserum B as the detection antibody. The bulk of immunoreactivity was found to elute at fractions 41–42 (Fig. 4), at a peak containing less than 5% of the total protein. Since this protein elutes last from the cation-exchanger, it was assumed that it must represent one of the most basic proteins of seminal plasma. Immunoreactive fractions 41–42 were preconcentrated approximately 100-fold by ultrafiltration and sepa-

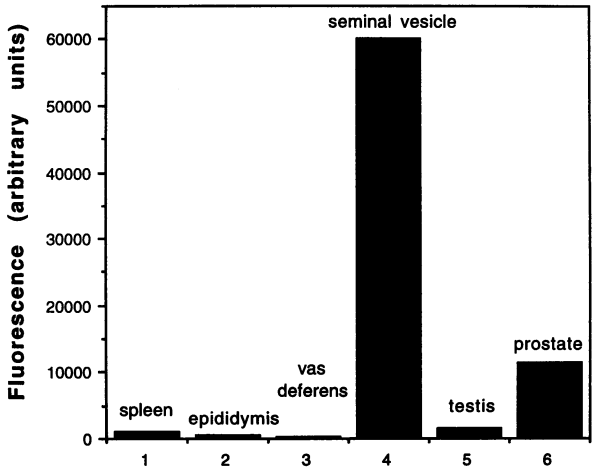

FIGURE 3. *Tissue specificity of the BRCA1-like immunoreactive protein. Tissue extracts from the tissues shown were analyzed; spleen extracts were used as the negative control.*

rated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. After protein transfer to nitrocellulose and probing with SG-11 antibody, four major bands with molecular weights of 110, 20, 14, and 12 kDa were obtained (data not shown).

The concentrated immunoreactive material from cation-exchange chromatography (fractions 38–44) was subjected to SDS-PAGE under reducing conditions and proteins were transferred to a PVDF membrane. The abovementioned four major bands were subjected to partial protein sequencing using the Edman degradation method. No sequence was obtained from the 110-kDa band, presumably because of N-terminally blocked protein. The bands at 20, 14, and 12 kDa gave the following sequences: HNKQEGRDHDK and NYKQEGRDHDK. Homology searching indicated that these sequences represent parts of semenogelin I and semenogelin II proteins. More specifically, these sequences correspond to a peptide previously identified in seminal plasma as having inhibin-like activity[15] and to a peptide known as the predominant basic protein of seminal plasma.[16] This peptide is produced by fragmentation of semenogelin at positions 85 and 136, as described by Lilja *et al.*[17] The fragment appears as a dimer with a molecular weight of 10 to 12 kDa.[16]

Our preliminary work indicated that the molecular weight of the major immunoreactive species of seminal plasma is between 300 and 600 kDa.[14] This was further verified in the present study with two different gel filtration columns (Fig. 5). The immunoreactive species at molecular weight 300 to 600 kDa was detected by assays using either SG-11 or the semenogelin-specific monoclonal antibody M9 for capture, suggesting that this macromolecule contains semenogelin. The peak at approximately 25 kDa, detected with capture antibody M9 (Fig. 5B), was also detected on Western blots (see below) and likely represents a semenogelin fragment.

Semenogelin Immunoreactivity

The semenogelin-specific antibody M9 and purified semenogelins were used to confirm that the seminal immunoreactive protein was semenogelin. Seminal plasma and semenogelin I and semenogelin II standards were analyzed using immunoassays that involved SG-11 or M9 as capture antibodies and antiserum B or D-20 as detection antibodies. Analysis performed by using the growth hormone mouse monoclonal antibody as the capture antibody served to control for nonspecific interactions. Data are presented in Figures 6 and 7. It was found that although SG-11 reacted effectively with a substance present in seminal plasma (Figs. 6A and 7A), it did not bind either semenogelin I or semenogelin II (Figs. 6B,C and 7B,C). Antibody M9 recognized both semenogelin I and II, as expected. This antibody could also be used as the capture antibody to monitor seminal plasma semenogelin concentrations (Figs. 6A and 7A). It was further shown that M9-captured semenogelins from seminal plasma (Fig. 7A) or purified semenogelins (Fig. 7B,C) were recognized by antibody D-20.

These data allow us to speculate that an assay comprising SG-11 antibody for capture and either antiserum B or D-20 for detection recognizes an immunoreactive species of seminal plasma that possesses a C-20 (SG-11) BRCA1 epitope not present in the semenogelin molecule and a D-20 BRCA1 epitope that is present in the semenogelin I and II molecules. Comparatively, semenogelin II reacts more strongly than semenogelin I with the D-20 antibody, since at the same concentration, it generates an approximately fourfold higher signal (Fig. 7B,C).

To further confirm that the immunoreactive seminal plasma protein represented semenogelin, we performed immunoblotting experiments using gel filtration fractions 23–30 (Fig. 5) as source of the immunoreactive protein. Data are shown in Figure 8A. Probing with the growth hormone monoclonal antibody (negative control) produced no bands, indicating absence of nonspecific effects (data not shown). Probing with antibody M9 identified a major band at 27 kDa and other bands at 20, 14, 12, and 6 kDa. These bands most likely represent semenogelin fragments containing the M9 epitope. Probing with SG-11 antibody revealed four major immunoreactive bands at 110, 20, 14, and 12 kDa. The latter three bands were those that were shown by microsequencing to contain the semenogelin sequence. Probing with D-20 antibody detected bands at 27, 23, 20, 14, 12, and 8 kDa. We

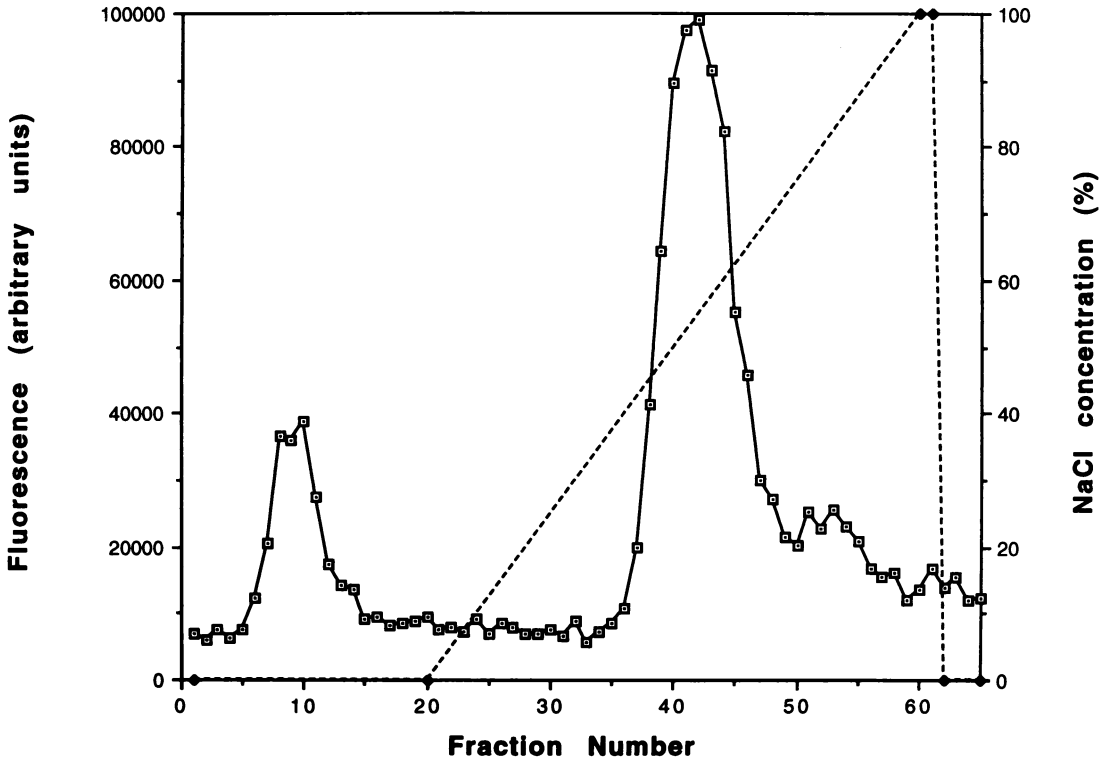

FIGURE 4. *Immunoreactivity of seminal plasma separated by ion-exchange chromatography. The peaks represent immunoreactivity using the assay configuration of Figure 1 with SG-11 as capture antibody and antiserum B for detection. Dotted lines indicate gradient elution as described in the text.*

further examined whether binding of these antibodies to the protein fragments on the blot could be blocked by peptide C-20 or D-20 (Fig. 8A). In accordance with the data of Figure 2, peptide C-20 blocked binding of SG-11 to the immunoreactive bands at 110, 20, 14, and 12 kDa. The binding was not affected when SG-11 was incubated with peptide D-20. Similarly, binding of D-20 antibody to immunoreactive proteins at 27, 23, 20, and 8 kDa was effectively blocked by peptide D-20 but not by peptide C-20. In contrast, neither peptide C-20 nor peptide D-20 affected binding of the M9 antibody.

The same experiment was performed by using purified semenogelin I as the protein source on the blots. As shown in Figure 8B, antibody M9 recognized full-length (66 kDa) semenogelin I and semenogelin fragments. This binding was not affected by peptides C-20 or D-20. Antibody D-20 reacted with intact semenogelin I, and the binding was blocked by peptide D-20 but not by peptide C-20. SG-11 antibody did not recognize semenogelin I on the blot, in accordance with data presented in Figures 6 and 7.

Macromolecular Complex

Analysis of the immunoreactive HPLC fraction 28 (Fig. 5, molecular weight ~300 kDa) by SDS-PAGE under reducing conditions revealed the presence of proteins with molecular weights varying from 110 to 6 kDa (Fig. 8A). This indicates that the assay of Figure 1 detects a macromolecular complex that contains in its structure the lower molecular weight SG-11 reacting proteins and the D-20 reacting proteins. This complex is effectively disrupted by dithiothreitol reduction during the Western blot procedure to release the smaller immunoreactive fragments. In support of this view is our finding of a greater than 90% decrease in the immunoreactivity of seminal plasma on dithiothreitol reduction (data not shown). Reduction breaks the complex into smaller proteins that do not possess both SG-11 and D-20 epitopes on the same molecule, as is the case with the macromolecular complex.

Homology Search

We searched for possible sequence homology between the peptides that are recognized by SG-11 antibody (peptide C-20) and D-20 antibody (peptide D-20) and either semenogelin I or semenogelin II. Using Blast-Align software (http://vega.crbm.cnrs-mop.fr), limited homology was observed between peptide C-20 and semenogelins I and II and slightly better homologies between peptide D-20 and semenogelins I and II (Fig. 9). In one area of peptide D-20, there were three identical sequen-

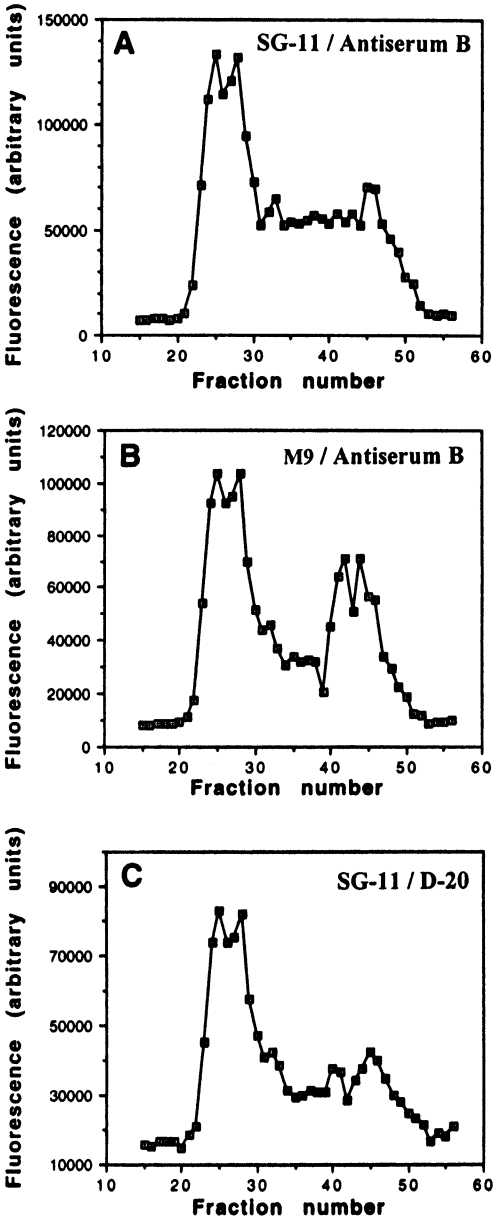

FIGURE 5. *Immunoreactivity of seminal plasma separated by gel filtration chromatography. The assays used for the analysis involved the following capture and detection antibodies: (A) SG-11/antiserum B; (B) M9/antiserum B; and (C) SG-11/D-20. Fractions 25–28 correspond to molecular weights of 300 to 600 kDa and fractions 42–44 to molecular weights of 20 to 30 kDa.*

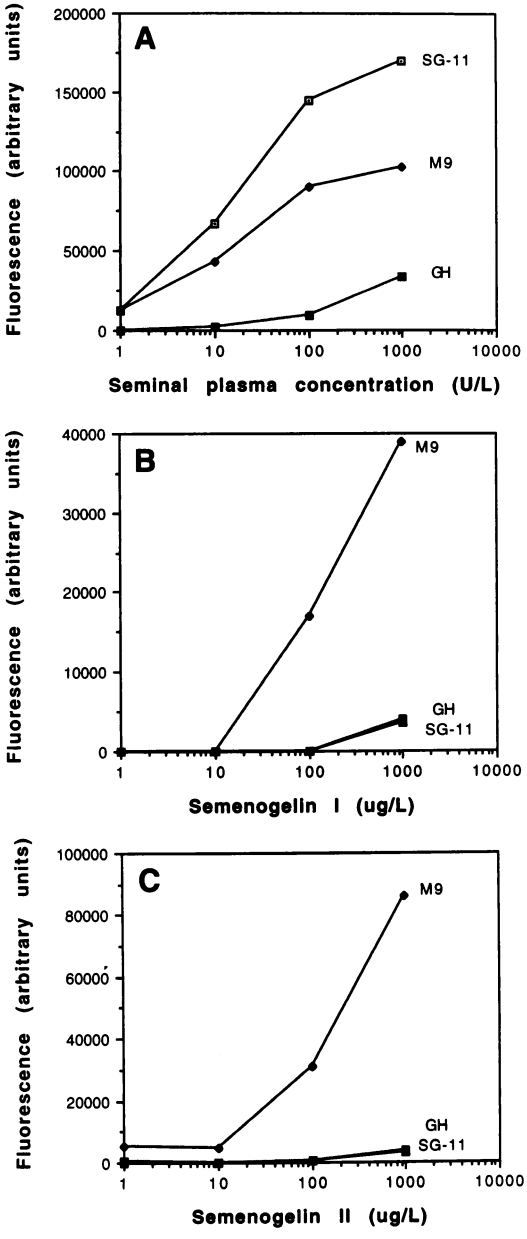

FIGURE 6. *Immunoreactivity of seminal plasma components and semenogelins I and II. The immunofluorometric assay was performed using SG-11, M9, or growth hormone antibody (GH) as the capture antibody and antiserum B as the detection antibody. (A) Standards were made from seminal plasma, and with purified (B) semenogelin I and (C) semenogelin II.*

tial amino acids (QNV) and in another area, four amino acids separated by a nonidentical amino acid (DL-AL). These data allow us to speculate that the observed cross-reactivity between D-20 antibody and semenogelins I and II may be due to these limited but significant homologies.

COMMENT

One of the major breakthroughs in breast cancer research has been the cloning of the two breast cancer susceptibility genes BRCA1 and BRCA2.[1] The subcellular localization of BRCA1 has been a matter of controversy, with various groups localizing it in the nucleus, the cytoplasm, or even outside of the cell, as a secreted protein. Part of this controversy arises because the immunologic reagents used for detection of BRCA1 protein are not well characterized. Wilson *et al.*[9] demonstrated that the polyclonal antibody C-20, specific for the C-terminal of BRCA1, reacts with endoplasmic growth factor receptor and HER2. The D-20 polyclonal anti-

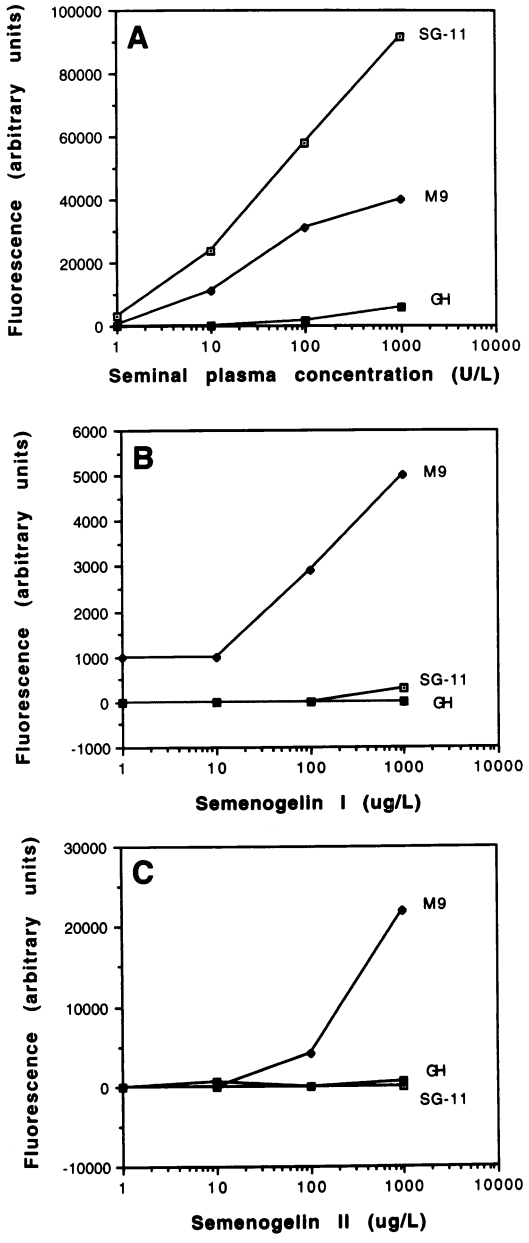

FIGURE 7. *Immunoreactivity of seminal plasma components and semenogelins I and II. The immunofluorometric assay was performed using SG-11, M9, or growth hormone (GH) as the capture antibody and D-20 as the detection antibody. Standard solutions were prepared as described in Figure 6.*

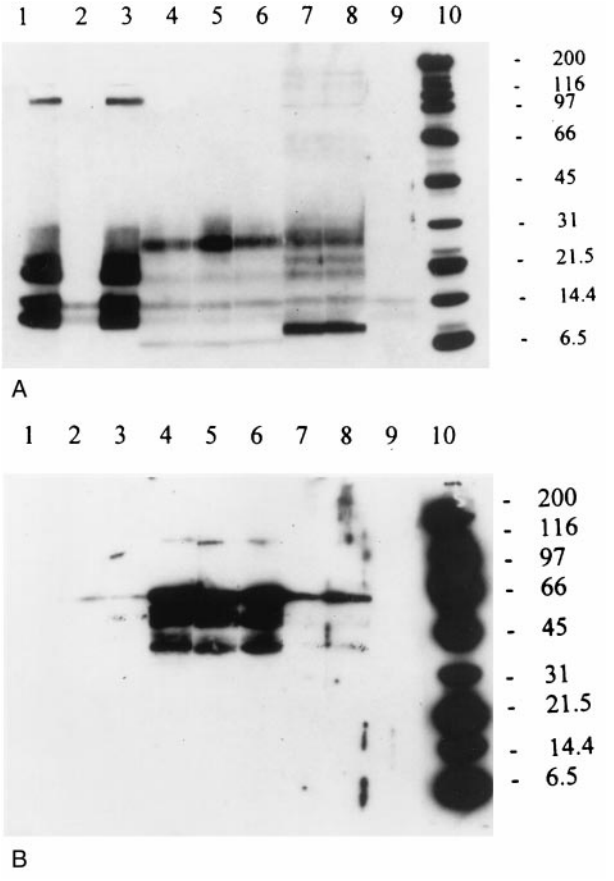

FIGURE 8. *(A) Specificity of BRCA1 antibodies for the seminal plasma immunoreactive compound. Gel filtration fraction containing the immunoreactive complex was used as the protein source on the blots. Antibodies used for probing were as follows. Lane 1: SG-11; lanes 2 and 3: SG-11 previously incubated with peptide C-20 or D-20, respectively; lane 4: M9; lanes 5 and 6: M9 previously incubated with peptide C-20 or D-20, respectively; lane 7: D-20; lanes 8 and 9: D-20 previously incubated with peptide C-20 or D-20, respectively; and lane 10: molecular weight markers. (B) Cross-reactivity of BRCA1 antibodies with semenogelin I. Purified semenogelin I was used as the protein source on the blots. Antibodies used for probing were as follows. Lane 1: SG-11; lanes 2 and 3: SG-11 previously incubated with peptide C-20 or D-20, respectively; lane 4: M9; lanes 5 and 6: M9 previously incubated with peptide C-20 or D-20, respectively; lane 7: D-20; lanes 8 and 9: D-20 previously incubated with peptide C-20 or D-20, respectively; and lane 10: molecular weight markers.*

body, specific for the aminoterminus of BRCA1, was shown to have no cross-reactivity with type I tyrosine kinase receptor, but the protein patterns detected with this antibody from cell extracts were found to be complex.[9] The monoclonal antibody SG-11, which recognizes the same region on BRCA1 as C-20, is believed to be free of cross-reactivities.[6]

We have recently reported that human seminal plasma contains a protein that immunoreacts strongly with antibodies SG-11 and D-20.[10] The most impressive finding of that study was the observation that protein detection in seminal plasma was possible even at seminal plasma dilutions as high as 10^6-fold. This prompted us to assume that SG-11 and D-20 must have high affinity for this seminal plasma protein and that this protein must be present at very high concentrations in seminal plasma. In the present study, we attempted to characterize this BRCA1-like immunoreactivity of seminal plasma.

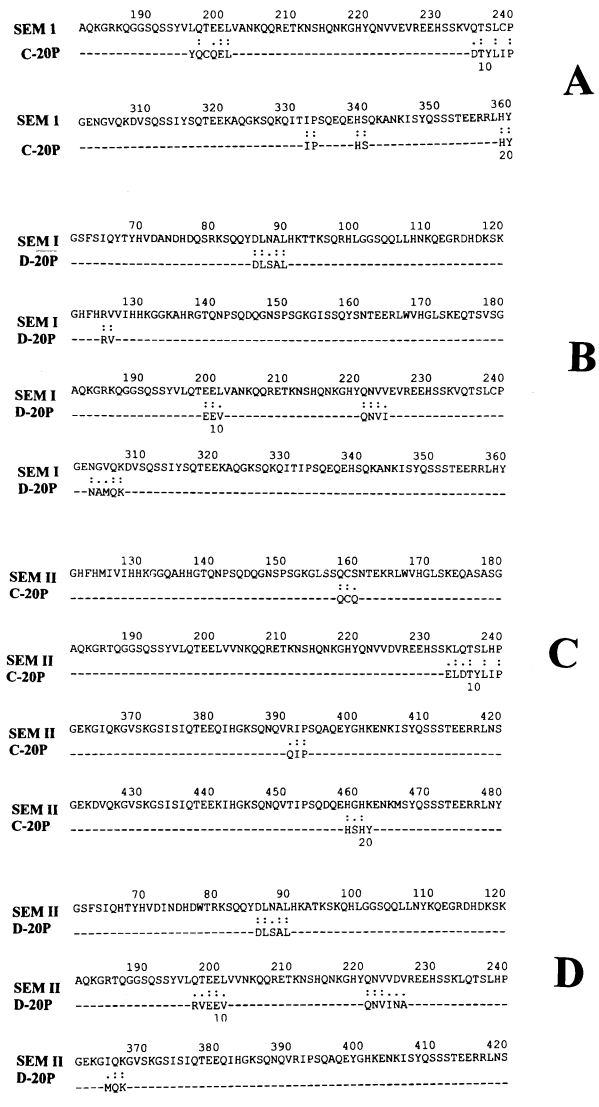

FIGURE 9. *Homology between peptides C-20 and D-20 and semenogelins I and II. Analysis was performed with Blast-Align software (http://vega.crbm.cnrs-mop.fr). Numbers refer to the sequence of semenogelin I (Accession P04279) and semenogelin II (Accession Q02383) as deposited in the Swiss-Prot data base (http://www.ncbi.nlm.nih.gov/irx/cgi-bin/swiss).*

Using immunologic assays, we first verified that antibodies SG-11 and D-20 capture the protein of interest through their binding site and not through nonspecific interactions. Antibody/protein binding was blocked by the specific peptide for each antibody. Unrelated peptides had no effect on binding, and BRCA1-irrelevant antibodies did not react with any seminal plasma components.

This BRCA1-like protein is produced by the seminal vesicles. Purification and sequencing of the immunoreactive protein indicated that it represents semenogelin I and II which are abundantly present in seminal plasma. Both immunoassays and immunoblotting indicated that the SG-11 antibody does not cross-react significantly with either purified semenogelin I or II, whereas the D-20 antibody recognizes both semenogelins. This cross-reactivity was effectively blocked by the D-20 peptide.

Using an immunoassay that recognizes semenogelins (M9 coating/antiserum B detection), we attempted to examine whether human nipple aspirate fluid, collected from human female breasts, contains semenogelins. We found no evidence that this fluid contains semenogelins.

Peter *et al.*[18] have recently demonstrated that semenogelin I and II interact noncovalently and by disulfide bridges to form a coagulum on ejaculation. Prostate-specific antigen-digested semenogelins produce fragments that can be cross-linked into complexes by factor XIIIa. We believe that the immunoreactive protein measured by our assay represents these macromolecular complexes. Indications to support this are as follows: (1) the seminal plasma immunoreactivity is diminished after reduction of disulfide bridges by dithiothreitol (a procedure that disrupts the complex) and (2) on separation of seminal plasma with gel filtration, the immunoreactive complex appears to have a molecular weight greater than 200 kDa. However, after reduction followed by SDS-PAGE, the major bands have low molecular weights (10 to 20 kDa). These bands are recognized by both SG-11 and D-20 antibodies on Western blots, and on protein sequencing, they are shown to represent semenogelin fragments.

This macromolecular complex, which is primarily held together by disulfide bridges, contains an SG-11 epitope and a D-20 epitope. The D-20 epitope is part of the semenogelin molecule. The SG-11 epitope, however, was not found on semenogelins by either immunoassay, Western blot, or homology search. It is thus speculated that the SG-11 epitope may be linked to semenogelin fragments but it is not part of their protein sequence. Porta *et al.*[19] reported that spermidine, a naturally occurring polyamine in human semen, is incorporated into peptides of about 10 to 20 kDa when added to human seminal plasma. These peptides are likely semenogelin fragments.[18] The speculation that the SG-11 epitope is linked to semenogelin fragments and the nature of this epitope need further investigation.

Homology studies indicated restricted sequence homology between the C-20 peptide and semenogelins I and II. The more substantial homologies observed between the D-20 peptide and semenogelins I and II may explain the cross-reactivity of the D-20 antibody with semenogelins.

In conclusion, we have partially characterized the cross-reactivity of seminal plasma to BRCA1 antibodies SG-11 and D-20. It was shown that both

epitopes of these antibodies appear on a macromolecular complex containing semenogelin fragments. The D-20 epitope is part of the semenogelin molecules, but the SG-11 epitope appears to be bound to semenogelin fragments. The limited homology and the sharing of immunoreactive epitopes between BRCA1 termini and semenogelins have no known biologic relevance at present. This study suggests that cross-reactivities of BRCA1 antibodies with other proteins should be considered until highly specific reagents for BRCA1 become available.

ACKNOWLEDGMENT. To Dr. Koichi Ishikawa for performing protein sequence analysis.

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