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Research Article

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Antigens and Antibodies Cross-Reactive to the Murine Mammary Tumor Virus in Human Breast Cyst Fluids

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ABSTRACT Human breast cyst fluids were shown to contain low concentrations of IgA (15–78 μg/ml) and IgG (33–145 μg/ml). The IgA:IgG ratios in individual breast cyst fluids ranged from 1:0.6 to 1:4. These levels are considerably higher than their ratio in serum (1:7). IgA from 33% of the 40 fluids examined, and IgG from 10% of the fluids, reacted with the murine mammary tumor virus (MuMTV). The reactivity was detected by an enzyme-linked immunosorbent assay that measures antibody binding to both the envelope glycoprotein and core protein of the virus. In a second series of experiments, IgA from 28% of 40 breast cyst fluids reacted only with MuMTV while IgA from 30% of the fluids was reactive with both MuMTV and the Rauscher murine leukemia virus. Antigen reactive with antiserum to the 28,000-dalton MuMTV core protein (p28), was also identified in a 165,000-g pellet fraction from breast cyst fluids. In individual fluids, the extent of IgA binding to MuMTV was positively correlated ($P \leq 0.01$) with the binding of anti-p28 antibody to the pellet of the breast cyst fluid. Fractions with the buoyant density of retroviruses (1.16–1.18 g/ml) or their cores (1.21–1.25 g/ml) were isolated from breast cyst fluids. These fractions contained a DNA polymerase capable of utilizing the reverse transcriptase-specific template, $dG_{12-18} \cdot poly$ rC^m. In addition, they reacted with antiserum to MuMTV p28 but not with antiserum to the 30,000-dalton Rauscher murine leukemia virus core protein.

INTRODUCTION

Benign mammary cystic disease is the most common breast lesion. It occurs in ~10% of women (1). Its prevalence in premenopausal women has led to the supposition that cyst formation is due to ovarian hormone stimulation (1); the actual etiological and pathogenic basis, however, remains unknown. Al-

though not universally accepted, several studies have demonstrated that women who have had benign mammary cysts are at an increased risk for the subsequent development of mammary adenocarcinoma (2–4).

The mammary cysts are filled with fluid. Treatment usually consists of needle aspiration of the fluids; cysts rarely recur at the site of aspiration. The cyst fluid differs significantly from serum in composition and probably arises as an exudate from epithelial cells lining the cyst (5, 6). The major proteins in breast cyst fluids are (*a*) a progesterone binding glycoprotein (6, 7), (*b*) Zn-alpha$_2$-glycoprotein, and (*c*) an unidentified protein with 15,000 mol wt immunologically unrelated to plasma proteins (6). In addition, a carcinoembryonic antigenlike protein has been identified in each of the 23 cyst fluids examined by Fleischer et al. (8). Immunoglobulin and complement components have not previously been quantitated in breast cyst fluids.

Antibodies (9, 10) or lymphocytes (11) reactive with murine mammary tumor virus (MuMTV)[1] or antigens reactive with MuMTV-specific antisera (12) have been demonstrated in breast cancer patients. To further examine possible relationships between benign mammary cystic disease and mammary adenocarcinoma, we have analyzed breast cyst fluids for evidence of components related to MuMTV.

METHODS

Material. Aspirated breast cyst fluids were obtained by the physicians of the Breast Service at Memorial Hospital.

The fluids were centrifuged at 10,000 g for 20 min, divided into aliquots, and stored at $-70°C$.

MuMTV (13) and rabbit or goat antiserum to the 28,000-dalton MuMTV core protein (p28), or to the Rauscher murine leukemic virus (R-MuLV) 30,000 dalton core protein (p30), were prepared as previously described (14). In pilot experiments utilizing 0.5 μg MuMTV or R-MuLV protein, it was determined that a 1:180 dilution of the goat anti-MuMTV p28 serum reacted with MuMTV to give an absorbance of 0.80 and with R-MuLV to give an absorbance of 0.11, as determined by our enzyme-linked immunosorbent assay (see below). Goat anti-R-MuLV p30 serum yielded absorbances of 0.124 and 1.346 with MuMTV and R-MuLV, respectively. The rabbit antisera exhibited similar titers.

Immunochemical analyses. The concentration of IgG, IgA, Clq, and C3 in breast cyst fluids were determined by the method of Mancini et al. (15).

Enzyme-linked immunosorbent assay (ELISA). Details of our ELISA assay for the determination of antibodies reactive with MuMTV fixed to wells of a microtiter plate have been published (10). Immunoglobulin binding was quantitated with alkaline phosphatase-conjugated swine anti-human IgG or IgA (heavy chain-specific, Orion Diagnostics, Helsinki, Finland), or alkaline phosphatase-conjugated goat anti-rabbit IgG, or rabbit anti-goat IgG (Microbiological Associates, Walkersville, Md.), by determining the absorbance of the solution at 400 nm after the addition of p-nitrophenyl phosphate. Duplicate determinations differing by >10% were reassayed. IgG binding was proportional to MuMTV concentration over a 0.1–10-μg viral protein range. Antibody to MuMTV membrane components (gp 52, gp 34) and to the major internal protein (p28), all were measurable by this assay. MuMTV antibody diluted as much as 1:3,000 was readily detectable (10).

Isolation of the MuMTV-related component. Breast cyst fluid was thawed, centrifuged at 10,000 g for 10 min, and the supernate centrifuged at 160,000 g for 60 min (SW 50.1 rotor) or 100,000 g for 90 min (SW 27 rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The resultant pellet was resuspended in phosphate-buffered saline (PBS), layered over an 18% (wt/vol) solution of metrizamide (Accurate Chemical and Scientific Corp., Hicksville, N. Y.) and again centrifuged at either 100,000 g or 160,000 g. The pellet was resuspended in PBS, layered over a 5 ml 15–60% (wt/wt) gradient of sucrose in PBS and centrifuged for 16 h at 165,000 g. The gradient was fractionated and density determined from refractive indices. The concentration of material having the buoyant density of MuMTV (1.18 g/ml) or MuMTV cores (1.21–1.25 g/ml) was achieved by pooling the relevant fractions, diluting to <10% sucrose with PBS and centrifuging at 165,000 g for 60 min.

DNA polymerase assays. Aliquots of the sucrose gradient

TABLE I
Immunoglobulin and Complement Levels in BCF

Component	No. positive*	Concentration‡ (range)
IgG	26	83.1 μg/ml (33.0–145.0)
IgA	24	40.4 μg/ml (15.0–78.0)
IgM	2	Not done
Clq	5	2.1 μg/ml (1.3–4.2)
C3	1	Not done

* 33 BCF were analyzed by immunodiffusion.
‡ Mean concentration of the positive samples.

TABLE II
BCF IgA or IgG Binding to MuMTV

Sample No.	Absorbance (400 nm)*	
	IgA	IgG
1	0.026	0
2	0.025	0
3	0.074	0
4	0	0.181
5	0.080	0
6	0.025	0
7	0.058	0
8	0.025	0
9	0.009	0.038
10	0.046	0
11	0.052	0
12	0.037	0
13	0	0.184
14	0.119	0
15	0.075	0
16	0	0.055
17	0.038	0
18–40	0–0.012	0

* A background absorbance of 0.100 has been subtracted from all values.

fractions were assayed for dG·rC^m-templated DNA polymerase activity using reaction conditions optimal for MuMTV reverse transcriptase (16). The reaction mixture, final volume 100 μl, contained: 0.13 μmol MnCl$_2$, 0.1% Triton X-100, 0.10 μmol dithiothreitol, 0.02 μmol ATP, 0.5 μg dG$_{12-18}$·poly rC^m (P-L Biochemicals, Inc., Milwaukee, Wisc.), 0.5 μCi [^{3}H] deoxy guanosine triphosphate (dGTP) (10.0 Ci/mmol, New England Nuclear, Boston, Mass.), and 0.1 μmol Tris-HCl, pH 7.5. ATP was added to specifically inhibit any terminal deoxynucleotidyl transferase that may be present (17). Incubation was at 37°C for 60 min. Quantitation of the acid-insoluble product formed was as described (9).

RESULTS

Immunoglobulins and complement in breast cyst fluid. Fluids from 33 patients were analyzed by immunodiffusion for IgG, IgA, IgM, Clq, and C3. The results are presented in Table I. Low concentrations of IgA (15–78 μg/ml) and IgG (33–145 μg/ml) were found in most of the samples. The IgA:IgG ratios in individual breast cyst fluids ranged from 1:0.6 to 1:4; the IgA:IgG ratio in human sera is ~1:7. In contrast, only two samples had detectable levels of IgM; one contained C3, and five had Clq. The absence of some serum components and an increased IgA:IgG ratio in breast cyst fluid suggests that IgA probably is produced locally in the vicinity of the cyst.

IgA and IgG binding to MuMTV or R-MuLV. We next sought to determine whether breast cyst fluid immunoglobulins would react with MuMTV. MuMTV (0.5 μg protein) was fixed to individual wells of a microtiter plate, incubated with 1:5 dilutions of 40 breast

TABLE III
Reactivity of BCF IgA with MuMTV or R-MuLV

Sample No.	Absorbance (400 nm)*	
	MuMTV	R-MuLV
1	0.365	0
2	0.023	0
3	0.188	0
4	0.067	0
5	0.947	0
6	0.367	0
7	0.087	0
8	0.102	0
9	0.553	0
10	0.195	0
11	0.356	0.004
12	0.027	0.492
13	0.095	0.091
14	0.230	0.213
15	0.587	0.728
16	0.144	0.085
17	0.051	0.017
18	0.165	0.302
19	0.068	0.085
20	0.262	0.245
21	0.498	0.100
22	0.216	0.338
23	0.113	0.327
24–40	0	0

* A background absorbance of 0.100 has been subtracted.

cyst fluids; and IgA or IgG binding to the virus was quantitated by the ELISA assay with alkaline phosphatase-conjugated antisera. The results are shown in Table II. Human sera diluted to reflect breast cyst fluid IgG or IgA concentrations yielded absorbances of up to 0.10 in our assay. This value (0.10) was therefore chosen as the boundary between specific and nonspecific antibody reactivity. IgA from 33% of the fluids (13/40) yielded an absorbance ≥ 0.025 over background. IgG from 10% of the fluids (4/40) also exhibited binding to MuMTV. No fluid was positive for both IgA and IgG binding. The extent of IgA binding to MuMTV was not related to the IgA concentration in the cyst fluid, thereby making it improbable that we were merely measuring nonspecific binding.

The specificity of IgA binding to MuMTV was next examined. MuMTV or R-MuLV were fixed to microtiter plate wells, incubated with 1:5 dilutions of 40 breast cyst fluids and IgA binding to both viruses was quantitated. The results are shown in Table III. IgA from 28% of the fluids (11/40) reacted with MuMTV but not with R-MuLV, 30% (12/40) reacted with both viruses and 42% (17/40) were unreactive with either virus; no fluid reacted solely with R-

MuLV. The final observed absorbances in this experiment were higher than those in Table I due to the use of a new preparation of alkaline phosphatase-conjugated antisera and its utilization at a lower dilution. Titration curves of IgA binding to MuMTV are shown in Fig. 1.

Anti-MuMTV p28 serum reactivity with breast cyst fluids. Individual breast cyst fluids from 17 women were centrifuged at 10,000 and 165,000 g. The high speed pellet fraction was resuspended in PBS, layered over 18% metrizamide, and recentrifuged. The final pellets were each suspended in carbonate buffer, pH 9.8, and distributed to four wells of a microtiter plate. Following incubation at 4°C for 18 h, the wells were washed with PBS-0.05 Tween 20 and assayed by our ELISA assay for reactivity with serum from either an unimmunized rabbit or from a rabbit immunized with MuMTV p28. If present, retroviruses or their cores would be found in the metrizamide pellet fraction.

The results of these experiments are shown in Fig. 2. The extent of anti-p28 serum binding to the pellet fraction minus the binding of normal serum to the fractions (0.05–0.17 absorbance) is plotted for each individual fluid. Reactivity of p28 antiserum with the fixed fractions was evident in 41% (7/17) of the samples (absorbance 0.1). Furthermore, the extent of binding was correlated ($P \leq 0.01$) with the binding capacity of IgA from a particular fluid to MuMTV (Fig. 3).

Characterization of the breast cyst fluid MuMTV-like component. To determine if further similarities existed between MuMTV and breast cyst fluid components, fluids were fractionated according to procedures utilized to purify retroviruses and their
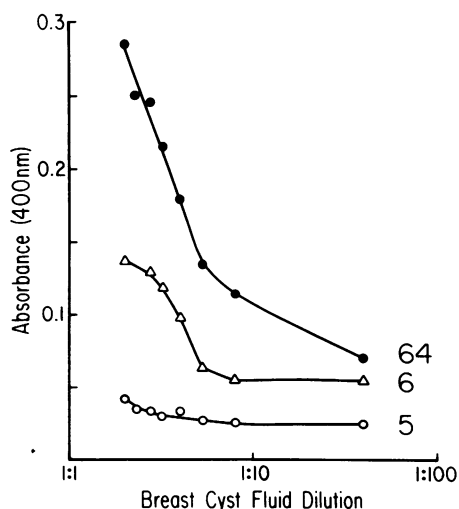

FIGURE 1 Titration of IgA reactive with MuMTV in BCF. Three BCF were diluted in PBS-Tween and assayed by the ELISA assay for IgA binding to MuMTV.

cores from biological fluids. Intact retroviruses have a buoyant density of 1.16–1.18 g/ml, while the viral cores (nucleoids) band at 1.21–1.25 g/ml. Within the cores are RNA and RNA-instructed DNA polymerase (reverse transcriptase).

Breast cyst fluids from 11 individual women were fractionated by velocity centrifugation, pelleting through 18% metrizamide and sucrose buoyant density centrifugation, and assayed for DNA polymerase activity using the synthetic RNA templates dT_{12-18}·poly rA or dG_{12-18}·poly rC^m. Results for two fluids using the latter template are shown in Fig. 4. MuMTV was also analyzed in a parallel gradient. MuMTV reverse transcriptase was able to utilize dG_{12-18}·poly rC^m; activity was localized mainly at the density of intact virus (1.17 g/ml) with some activity also evident at core densities (1.21–1.25 g/ml). Both cyst fluids also exhibited dG_{12-18}·poly rC^m activity in fractions corresponding to the core density; in one case, fluid 77, activity was also present at the density of intact virus. Each of the 11 fluids exhibited DNA polymerase activity in the viral and/or core regions.

ATP was included in our assays to specifically rule out that the observed activity was due to terminal deoxynucleotidyl transferase. We had previously observed that this enzyme was present in some breast cyst fluids but that it was always localized at the top of the sucrose gradients and not in the virus or core regions (18).

FIGURE 3 Relationship between BCF IgA binding to MuMTV and p28 anti-serum binding to BCF. IgA binding to MuMTV (Table III) vs. anti-p28 serum binding to the 165,000-g pellet fraction (Fig. 2) were plotted for individual BCF.

FIGURE 2 Reactivity of rabbit antiserum to MuMTV p28 with 165,000 g fractions from BCF. Fractions were prepared from individual BCF (Methods) and fixed in quadruplicate to wells of a microtiter plate. Two wells of each sample were incubated with sera from an unimmunized rabbit and two wells were incubated with rabbit anti-p28 serum. IgG binding was quantitated by the ELISA assay and the extent of normal serum binding (0.05–0.17 absorbance) was subtracted from the anti-p28 binding.

Sucrose gradient fractions from six breast cyst fluids were also analyzed for reactivity with antisera to the MuMTV or R-MuLV core proteins. The fractions were concentrated by centrifugation, fixed to wells of a microtiter plate and analyzed by the ELISA assay (Table IV). Purified MuMTV was strongly reactive with antiserum to MuMTV p28 and only weakly reactive with R-MuLV p30 antiserum. The extent of reactivity of the p28 antiserum with R-MuLV (0.221) was chosen as the boundary between specific and nonspecific reactivity. An absorbance of 0.190 resulted from the interaction of p30 antiserum with MuMTV; interaction of p30 antiserum with R-MuLV gave an absorbance of 0.880. Fractions with the density of viral cores were significantly more reactive with MuMTV p28 antiserum than with R-MuLV antiserum in four of five samples; two of five fractions with the density of the virus also exhibited preferential reactivity to the MuMTV antiserum.

DISCUSSION

Low concentrations of IgA and IgG were identified in most breast cyst fluids. The absence of other serum components from breast cyst fluids plus a higher IgA:IgG ratio than that found in serum suggested
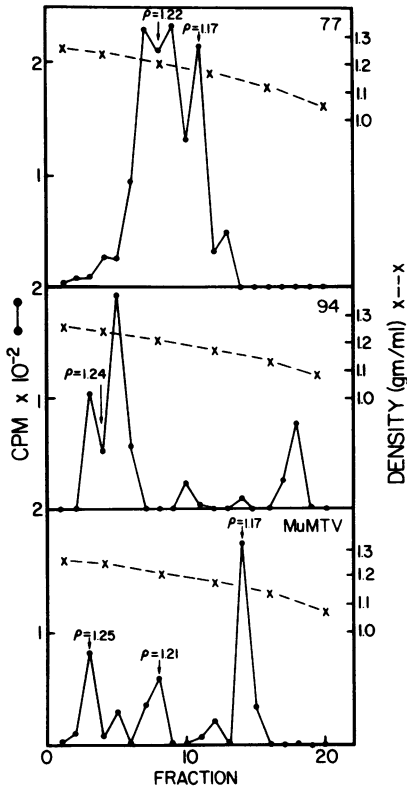
FIGURE 4 Buoyant density centrifugation and reverse transcriptase analysis of BCF 165,000-g pellet fraction. MuMTV, or the 165,000-g pellet fraction from cyst fluids, were centrifuged in 15–60% sucrose gradients for 16 h, fractionated, density determined from refractive indices, and aliquots assayed for reverse transcriptase activity using [^{3}H]dGTP and dG$_{12-18}$·poly rC^m.

erythrocytes, or carbohydrates present in cell membranes.

If the IgA that reacted with MuMTV was produced locally, then an antigen cross-reactive with MuMTV might also be expected to be present in the cyst fluid. The antigen reacting with antiserum to the MuMTV core protein, p28, was in fact located in a 165,000-g pellet fraction from 41% of BCF. The extent of p28 antiserum binding to the cyst fluid pellet fraction was proportional to the reactivity of IgA in a particular fluid for MuMTV. This is consistent with a mechanism whereby IgA antibody is produced locally in response to the presence of an MuMTV-related antigen. Additional similarities between MuMTV and a component of BCF were that both were present in the 10,000-g supernate, 165,000-g pellet fractions, pelleted by centrifugation through 18% metrizamide and contained a DNA polymerase able to utilize dG$_{12-18}$·poly rC^m in fractions possessing buoyant densities of 1.18 to 1.25 g/ml. All available data suggest that dG$_{12-18}$·poly rC^m is transcribed only by reverse transcriptase (16). Cyst fluid-derived components were also shown to preferentially react with antiserum to MuMTV p28 over antiserum to R-MuLV p30.

It might be useful to estimate the concentration of that IgA was probably produced by cells in the vicinity of the cyst.

Binding of these immunoglobulins to MuMTV was then examined. IgA in breast cyst fluids was more reactive with MuMTV than was IgG. Specificity of the IgA for MuMTV was determined by comparing binding to MuMTV and to R-MuLV. Of 58% (23/40) of breast cyst fluids that contained IgA reactive with MuMTV, half (12/23) were also reactive with R-MuLV while the remainder (11/23) were specific for MuMTV (Table III). Thus, 28% (11/40) of the breast cyst fluid (BCF) had IgA reactive only with MuMTV. These results indicate that there are at least two classes of BCF IgA, one reactive with both murine type B and type C viruses, and a second specific for type B MuMTV. Experimentation is now in progress to further define the IgA specificity for MuMTV. Initial results indicate that the reactivity is not removed by incubation of the BCF with fetal calf serum, sheep

TABLE IV
Reactivity of BCF Sucrose Gradient Fractions with Antiserum to MuMTV p28 or R-MuLV p30

Sample	Density	Absorbance (400 nm)*	
		anti-p28	anti-p30
	g/m		
BCF 29	1.23	0.441	0.193
BCF 29	1.18	0.148	0.152
BCF 65	1.23	0.325	0.181
BCF 65	1.18	0.129	0.150
BCF 68	1.23	0.171	0.181
BCF 68	1.18	0.349	0.116
BCF 70	1.23	0.419	0.242
BCF 70	1.18	0.110	0.126
BCF 80	1.19	0.439	0.062
BCF 81	1.23	0.277	0.130
MuMTV	1.19	0.912	0.221

165,000-g pellet fractions from 1.5–3.5 ml BCF were centrifuged to equilibrium in 15–60% sucrose gradients. Fractions with viral or viral core densities were pooled, concentrated by centrifugation, fixed to wells of a microtiter plate, and assayed by the ELISA method for reactivity with goat antisera to the MuMTV or R-MuLV core proteins. Antisera titers are given in Methods.
* An absorbance >0.221, the reactivity of anti-p30 serum with MuMTV, is considered positive.

MuMTV-equivalents in BCF. From the data in Table IV, 0.5 μg of bound MuMTV yielded an absorbance of 0.912 in a reaction with p28 antiserum. If we assume that the absorbance varies proportionally with viral concentration, as is in fact the case (10), it can be calculated that the six fluids reactive with p28 antiserum contained 0.18–0.24 μg MuMTV equivalent per 3 ml fluid.

Low concentrations of particles with the morphology (19) and biochemical properties (20) or retroviruses have also been observed in some samples of human milk. They may be identical to the BCF components, since both are probably derived from mammary epithelia. Schlom et al. (20) have determined, based on nucleic acid hybridizations, that there are up to 4×10^7 viruslike components/ml of human milk. If we estimate that a single retrovirus component is 7×10^{-10} μg, then we can calculate that there may be up to 0.03 μg virus/mg milk. This is comparable to the 0.06–0.08 μg components/ml we estimated to be present in BCF (see above). Whether these components originate from exogenous infection or represent expression of endogenous genetic information, is still unclear.

Mesa-Tejada et al. (12) reported that paraffin sections of 119 benign breast lesions were unreactive with antibody to MuMTV. This difference from our results may possibly be due to the greater accumulation of MuMTV-related antigen in the cyst fluids than in the surrounding tissue. Furthermore, components related to the MuMTV core protein, p28, have, to our knowledge, not been detected in human breast cancers. A p28-like protein has been identified, however, in human milk particles (21).

Because only a fraction of the 35% of women with detectable MuMTV-related components in their cyst fluids would be expected to develop mammary adenocarcinoma, it appears that the presence of these components is not sufficient by itself to induce malignancy. Perhaps additional genetic and/or environmental factors are needed to complete the transformation process. Evaluation of potential deleterious or beneficial consequences of the appearance of these components in the mammary gland awaits further characterization of their immunological, biochemical, and biological properties.

ACKNOWLEDGMENTS

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