Circulating Immune Complexes in Sera of Dogs with Benign and Malignant Breast Disease


ABSTRACT

The Raji cell radioimmunoassay was adapted to measure circulating immune complexes (CIC's) in the sera of 74 dogs with benign and malignant breast disease. In dogs with recently diagnosed spontaneous breast cancer, 57% (29 of 51) had elevated CIC's, with some levels as high as those found in the sera of dogs with systemic lupus erythematosus. The sera of 10 of 23 dogs with benign breast disease also demonstrated elevated levels of CIC's. Two weeks following mastectomy, repeat CIC levels were obtained in 30 dogs. Elevated CIC levels returned to normal in all dogs with benign breast disease but in only 33% of dogs with breast cancer. Dogs with persistent elevation of CIC's were at greater risk of developing metastatic breast cancer. Serum total hemolytic complement was significantly higher (<0.05) in dogs with untreated breast cancer than in healthy dogs but did not correlate with the level of CIC's found. Two weeks after mastectomy, total hemolytic complement levels had returned to normal. By sucrose density gradient ultracentrifugation, the complexes were shown to sediment at 19 S. These studies indicate that the dog may be a good model for elucidating the significance of elevated CIC's in breast cancer.

INTRODUCTION

CIC's have been found in a variety of human neoplasms when measured by techniques such as the Raji cell radioimmunoassay, C1q binding test, and C1q deviation test (4, 13, 17, 18). Sera from human breast cancer patients frequently contain elevated levels of CIC's. Hoffken et al. (4), using a C1q binding test, reported that all 22 sera of women tested with breast cancer had elevated levels of CIC's prior to mastectomy. Theofilopoulos et al. (18) studied 44 breast cancer patients and found 15 of 44 to have elevated complexes by the Raji cell radioimmunoassay. Rossen et al. (13), using a C1q binding technique, found elevation of CIC's in 20 of 27 patients. In 91 patients reported by Teshima et al. (17), levels of CIC's were 3-fold higher in sera of breast cancer patients than in sera of healthy individuals.

Despite these studies, the use of CIC's for monitoring the course of malignant disease is uncertain. The long natural history of human breast cancer with the inherent difficulties in follow-up make an animal model attractive to clarify the relationship between immune complexes and the course of the disease. The similarities between human and canine breast cancer make the dog a useful experimental model in the study of this tumor (2, 14). Spontaneous breast cancer is the most common cancer in female dogs with an incidence of 105 cases/100,000 dogs at risk (14). The pathology including lymph node spread is similar to that of breast cancer in the human. The disease often spreads systemically, with bone, lung, and liver being frequent sites of metastases. An additional advantage is that the natural course of the disease is usually manifested within 2 years in the dog as compared to 5 years, 10 years, or longer in humans.

The determination of CIC levels in dog serum has been reported previously (12, 15). Terman et al. (15), using the C1q binding test, showed that 4 of 4 sera of dogs with breast cancer had elevated levels of CIC's. Poskitt and Poskitt (12), using the binding of CIC's to the Fc receptor of the L1210 murine leukemia cell, studied 20 normal dogs and 16 dogs with systemic lupus erythematosus. None of the sera from normal dogs exhibited binding, while 10 of 16 sera from dogs with systemic lupus erythematosus demonstrated binding to the L1210 cell.

In the present study, the Raji cell radioimmunoassay was adapted to measure CIC's before and after mastectomy in the sera of dogs with benign and malignant breast disease.

MATERIALS AND METHODS

Patient Population

Fifty-one dogs with spontaneous breast cancer and 23 dogs with benign breast disease were studied prior to mastectomy at the Animal Medical Center of New York. Follow-up studies were done 2 weeks after mastectomy in 30 dogs and 3 months after mastectomy in 22 dogs. The average age of the dogs with breast cancer was 9.5 years (range, 7 to 13 years). The average age of the dogs with benign breast disease was 10 years (range, 7 to 13 years). Twenty healthy female dogs with an average age of 9 years (range, 6 to 13 years) were used as controls. These dogs were randomly selected without regard to breed. Two dogs with systemic lupus erythematosus were also studied. The criteria for the diagnosis of canine systemic lupus erythematosus was polysystemic disease with an elevated titer of anti-nuclear antibodies.

Serum Samples

The blood was allowed to clot at room temperature for 1 hr and was centrifuged at 200 x g at 4° for 10 min in a refrigerated centrifuge (Damon PR-600 IEC centrifuge, Fisher Scientific Co., Springfield, N. J.). Serum samples were aliquoted and stored at -70° and were used only once.

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Measurement of CIC's

CIC's were measured by a modification of the Raji cell radioimmunoassay (20) as follows.

Preparation of Aggregated Dog IgG. Dog globulin from Cohn Fraction II (Miles Laboratories, Inc., Elkhart, Ind.) was dissolved in 0.01 M sodium phosphate buffer (pH 7.5). This was chromatographically purified on a DEAE-cellulose 52 column. The protein in the peak concentrated to 6 mg/ml was shown to be IgG by immunoelectrophoresis using monospecific rabbit anti-dog IgG (Cappel Laboratories, Downington, Pa.). Purified IgG was heated in a water bath at 63°C for 20 min and then centrifuged (Sorvall RC2-B; Ivan Sorvall, Inc., Norwalk, Conn.) at 1000 x g for 90 min in order to remove large aggregates. The protein content off the top half of the supernatant was determined (6) and was adjusted to 4 mg/ml.

Radioiodination of Rabbit Anti-Dog IgG. Rabbit anti-dog IgG was radioiodinated with 125I (New England Nuclear, Boston, Mass.) by the chloramine-T method (8). The labeled anti-dog IgG was extensively dialyzed for 36 hr against 0.01 M sodium phosphate buffer (pH 7.5). The specific activity of the 125I rabbit anti-dog IgG was 0.05 to 0.2 μCi/μg of protein. The protein concentration was 0.3 mg/ml.

Assay Procedure. Raji cells were used after a 48-hr culture in MEM. The cells were washed 3 times in MEM, and viability was determined by the trypan blue exclusion test. An aliquot of 4 x 10^6 Raji cells was incubated (37°C, 45 min) with 25 μl of test sera at 1:4 dilution in MEM. After 3 washes in MEM, the samples were incubated (4°C, 45 min) with 100 μl of 125I rabbit anti-dog IgG. The cells were again washed 3 times, and the radioactivity in the pellet was counted in a Packard Instruments Auto-Gamma counter. Results were compared against a standard curve of aggregated dog IgG. Serial dilutions of aggregated dog IgG from 0 ng in 10 μl of MEM to 40,000 ng in 10 μl of MEM were incubated (37°C, 30 min) with pooled normal dog serum or human serum as a complement source. Since dog serum and human serum yielded similar standard curves, only pooled normal dog serum was subsequently used. Levels of CIC's were expressed as μg equivalents of aggregated dog IgG. For simplicity, we will refer to this as μg/ml. A standard curve using heat-inactivated dog serum (56°C, 30 min) was also tested.

Immunofluorescence Studies

Fluorescein isothiocyanate-conjugated goat anti-canine C3 was obtained from Cappel Laboratories. Raji cells (2 x 10^6 in 50 μl MEM) were incubated (37°C, 45 min) with 50 μl of pooled normal dog serum or heat-inactivated (56°C, 20 min) normal dog serum. The cells were washed 3 times in MEM and incubated with 100 μl of fluorescein isothiocyanate-conjugated goat anti-canine C3 for 30 min at 4°C. The cells were again washed in MEM. Wet preparations were read with a Leitz Dialux fluorescence microscope equipped with a 100-watt mercury light source. Slides were evaluated for percentage of stained cells and intensity of staining.

Isolation of Complexes by Sucrose Density Gradient Ultracentrifugation

Sera from 2 dogs with breast cancer, one dog with benign breast disease, and 2 healthy dogs were used. A 200-μl sample of each serum was subjected to sucrose density gradient ultracentrifugation (10 to 40%, w/w) at 100,000 x g for 15 hr (Beckman Model L5-65; Beckman Instruments, Inc., Fullerton, Calif.). 125I Clq (11 S), IgG (7 S), and IgM (19 S) were used as markers. Twenty fractions, each containing 15 drops, were collected. In another experiment, the above sera were dialyzed against glycine-HCl buffer (pH 2.5) and subjected to sucrose gradient ultracentrifugation. Every second fraction was tested for the presence of IgG by the Ouchterlony double immunodiffusion method. Immune complexes were determined by the Raji cell method following the addition of normal dog serum (1:4 dilution) as a complement source. The 125I Clq (11 S) marker was determined in a gamma counter. Each aliquot was checked for sucrose concentration by a refractometer to ensure comparability among the different samples.

Biostatistical Methods

A multiple comparison test (3) was used to compare the mean CIC and TCH50 values of the dogs with breast cancer and benign breast disease with those of the normal dogs. For the CIC values, a logarithmic transformation was first applied to the data. The Wilcoxon paired-sample test (21) was used to compare the pre- and postmastectomy CIC and TCH50 values.

RESULTS

Pathology. Table 1 lists the pathology at diagnosis in dogs with malignant and benign breast disease. A total of 74 dogs were investigated. Adenocarcinoma limited to the breast was the most frequent finding (35 dogs). In 4 dogs, there was lymph node spread, and 5 dogs presented initially with widespread metastatic disease. Adenoma was by far the most frequent benign pathological diagnosis (18 dogs).

TCH50 Levels. Using the one-sided Dunnett's test, the serum TCH50 levels of dogs with untreated breast cancer were significantly elevated (p < 0.05) when compared with serum levels of healthy dogs (Chart 1; Table 2). The mean ± S.D. of 51

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastitis or hyperplasia</td>
<td>5</td>
</tr>
<tr>
<td>Adenoma</td>
<td>18</td>
</tr>
<tr>
<td>Adenocarcinoma in situ</td>
<td>7</td>
</tr>
<tr>
<td>Localized</td>
<td>35</td>
</tr>
<tr>
<td>Positive lymph nodes</td>
<td>4</td>
</tr>
<tr>
<td>Metastases present</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
</tr>
</tbody>
</table>
dogs with breast cancer was $110 \pm 33$ compared to $20$ healthy dogs at $93 \pm 32$. TCH$_{50}$ decreased from $111 \pm 34$ to $90 \pm 21$ in $22$ dogs where follow-up sera were obtained $2$ weeks after mastectomy (Chart 2). In the $23$ dogs with benign breast tumors, serum TCH$_{50}$ prior to mastectomy was $100 \pm 25$. This was not significantly different from values for the $20$ healthy dogs.

**Levels of CIC's in Sera of Dogs with Benign and Malignant Breast Disease.** A typical standard Raji cell radioimmunoassay curve using normal dog serum and increasing concentrations of aggregated γ-globulin is shown in Chart 3. When normal dog serum is heat inactivated at $56^\circ$ for $30$ min, only a slight increase in $^{125}$I anti-dog IgG binding to the Raji cells occurred. The addition of monomeric IgG from $0$ to $4$ mg/ml resulted in minimal $^{125}$I anti-dog IgG binding to the Raji cells (data not shown).

The CIC values for dogs with benign and malignant breast disease were both significantly higher ($p < 0.01$ and $p < 0.05$, respectively) than those for sera of healthy dogs (Chart 4; Table 2). When the cutoff value for CIC levels is set at $25$ μg/ml, then only $10\%$ of the $20$ normal dogs had CIC values above this as compared to $43\%$ of the $23$ dogs with benign breast disease and $57\%$ of the $51$ dogs with breast cancer. Seven of $9$ dogs with either lymph node or systemic metastases had elevated levels of CIC's.

Repeat CIC levels were determined in $22$ dogs with breast cancer (Chart 5). Twelve dogs ($54\%$) had increased CIC levels preoperatively, with elevated CIC's present $2$ weeks after mastectomy in $8$ dogs. Metastatic breast cancer developed within $3$ months in $5$ of $8$ dogs with persistently elevated CIC levels, but in only one of $14$ dogs without increased CIC's after surgery ($p < 0.05$). Eight dogs with benign breast disease were studied prospectively (see Chart 5). All $3$ dogs with initially increased CIC levels were found to have normal levels $2$ weeks after mastectomy.

Several dogs had levels of CIC's in their sera comparable to the elevated levels of CIC's found in the sera of $2$ dogs with systemic lupus erythematosus (SLE) shown for comparison.

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>TCH$_{50}$</th>
<th>CIC's</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of dogs</td>
<td>Mean</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>$51$</td>
<td>$110^{a}$</td>
</tr>
<tr>
<td>Benign tumors</td>
<td>$23$</td>
<td>$100$</td>
</tr>
<tr>
<td>Normal dogs</td>
<td>$20$</td>
<td>$93$</td>
</tr>
</tbody>
</table>

$^{a} p < 0.05$, significantly elevated from normal one-sided Dunnett's test.

$^{b} p < 0.01$, significantly elevated from normal one-sided Dunnett's test.
DISCUSSION

The observation that sera of cancer patients often contain elevated levels of CIC’s has provoked many unanswered questions. An animal model to study the implications of CIC’s in cancer would be useful. The dog has been considered a good model for human breast cancer because of the many similarities between the tumors in both species. In this study, the Raji cell radioimmunoassay was adapted to measure CIC’s in dog sera. Elevated levels of CIC’s were demonstrated in the sera of dogs with both benign and malignant breast disease. TCH50 was not found to correlate with the CIC level. This is not surprising in view of the individual variability in both rate of complement synthesis and the ability of CIC’s to bind complement. Kinetic studies of complement synthesis and catabolism would be required to more fully define the relationship between complement and CIC level.

Theofilopoulos et al. (19), using human sera, have previously demonstrated that the Raji cell binds immune complexes predominantly through C3 and C3b receptors on the cell surface. We have obtained several lines of evidence for this in the dog. Using an immunofluorescence technique, C3 from normal dog serum was shown to bind to Raji cells. Further, using the Raji cell radioimmunoassay, aggregated dog IgG bound to the Raji cells only minimally except in the presence of complement which enhanced binding dramatically. By sucrose density gradient ultracentrifugation, we isolated CIC’s from sera of 2 dogs with breast cancer and from the sera of one dog with benign breast disease. The complexes sedimented at approximately 19 S and contained IgG. Fractions obtained from centrifugation of normal dog serum under identical conditions or fractions of serum of dogs with breast cancer using glycine buffer (pH 2.5) did not contain CIC’s, and IgG was restricted to the 7 S region.

The finding of elevated CIC’s in dogs with benign breast disease was of interest. Day has also noted elevated CIC’s in several women with benign breast tumors. Women who have had a biopsy for benign breast disease have about a 3-fold risk of subsequently developing breast cancer (11). Fibrocystic disease, fibroadenomas, and intraductal papillomas have all been reported to have potential for malignant transformation (5). The connection between benign breast disease and subsequent breast cancer in the dog has not been adequately studied. Only further follow-up studies of dogs with benign breast disease will determine whether the presence of elevated CIC’s is of prognostic importance.

The presence of elevated levels of CIC’s in both benign and malignant dog breast sera raises the question of whether a similar mechanism underlies their formation. This is of particular interest in view of several reports associating the mouse mammary tumor virus and antibodies to this virus with breast cancer in several species including humans (9, 10, 22). There is no information concerning a possible relationship between this virus and breast cancer in the dog.

Terman et al. (16) have recently reported necrosis of canine mammary carcinoma after extracorporeal perfusion of plasma over Staphylococcus aureus. The Cowans I strain of Staphylococcus aureus binds IgG and IgG-containing immune complexes. Our finding of elevated CIC levels in 57% of dogs with breast cancer may be of significance with respect to the study of Terman et al.

The stage or extent of disease has been correlated with the level of CIC’s in several reports (1, 4, 16, 18). Seven of 9 dogs
in this study with lymph node or systemic metastases had elevated CIC's. The dogs with persistent elevation of CIC levels appeared to be at greatest risk of developing metastatic breast cancer during the 3-month period after mastectomy. The short natural history of breast cancer in the dog should enable further clarification of the relationship between immune complexes and the clinical course of the disease.

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REFERENCES

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