Circulating Immune Complexes and Immunoglobulin M-Class Rheumatoid Factor in Rats Bearing Mammary Adenocarcinomas Which Vary in Ability to Metastasize

Dave B. S. Hoon, Barry Ziola, Svein Carlsen, Robert Warrington, and Ian Ramshaw

Departments of Microbiology [D. B. S. H., B. Z., S. C., I. R.] and Biochemistry [R. W.], University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0WO

ABSTRACT

In order to explore whether immune complex (IC) formation and immunoglobulin M-class rheumatoid factor (RF) synthesis are related to tumor progression, solid-phase enzyme immunoassays were used to test for ICs and RF in rats bearing three different syngeneic mammary adenocarcinomas. The mammary adenocarcinoma cell lines used produced either extensive metastasis (13762), metastasis in only a proportion of the animals given injections (R3230AC), or no metastasis (DMBAa). DMBAa and 13762 tumor-bearing rats developed only low levels of circulating ICs. Of 18 animals bearing R3230AC tumors, four developed palpable lymph node metastasis (macrometastasis), while another five showed evidence of metastasis only upon histological examination (micrometastasis). R3230AC tumor-bearing animals which did not develop metastasis were found to have significantly higher IC levels than those rats with metastasis. Several sera from rats bearing R3230AC tumors were fractionated by molecular sieve chromatography. Most of the ICs in these sera were 7S to 19S in size. Significant RF synthesis occurred only in rats bearing R3230AC tumors and only during terminal tumor growth. These results show that IC formation and RF synthesis varies in animals bearing different mammary adenocarcinomas.

INTRODUCTION

Circulating ICs are detectable in humans and animals bearing a variety of neoplasms (13, 16, 19). However, attempts to correlate IC levels with diagnosis or staging of neoplastic disease have met with little success (6). Results seem to vary not only with the type of neoplasm examined but also with the clinical stage of the disease. In retrospect, this finding is not surprising, since heterogeneity observed within tumors might be related to appearance of ICs in serum.

Only a few animal tumor models have been examined with regard to circulating ICs. Jennette and Feldman (8) studied formation of ICs in rats bearing Moloney sarcoma virus-induced tumors and found that rats with progressively growing tumors had higher levels of ICs than did animals in which tumors regressed. Circulating ICs have also been observed in rats bearing the chemically induced hepatoma D23 (1, 7). In these model systems, IC levels increased relative to tumor burden and then declined despite continued tumor growth (7, 8).

Because of the variable results that have been observed in studies of ICs using differing tumor systems, we attempted to determine whether IC formation was related to the ability of the tumor cell to metastasize. Rat mammary adenocarcinoma cell lines were chosen largely because clinical studies suggested that ICs are found in breast cancer patients (6, 13, 14, 18). Three types of rat mammary adenocarcinomas (13762, R3230AC, and DMBAa) were used, 2 (13762 and R3230AC) of which exhibit lymphatic metastases that occur with some types of malignant human breast tumors (4). A solid-phase C1q EIA was used for IC detection. In all cases, IC levels rose as the primary tumor progressed. However, a clear relationship between metastasis and IC levels was not found.

It has been suggested that RF synthesis is related to the formation of ICs (10, 20). Moreover, rheumatic disease and cancer may be temporarily related (15). For these reasons, we also measured RF levels in sera from the mammary adenocarcinoma-bearing rats. A solid-phase EIA was used for this purpose. RF synthesis occurred in animals bearing only one of the 3 adenocarcinomas and then only late in primary tumor growth.

MATERIALS AND METHODS

Mammary Adenocarcinomas. 13762 MAT-B (ascites), R3230AC (solid), and DMBAa (solid) rat mammary adenocarcinoma cell lines were obtained from Dr. A. E. Bogden (Mason Research Institute, Worcester, Mass.). Growth characteristics and other properties of these tumors have been documented (3). Briefly, the 13762 mammary adenocarcinoma is a chemically induced tumor that is poorly differentiated and exhibits extensive metastasis. The DMBAa mammary adenocarcinoma is a chemically induced, differentiated tumor cell line which exhibits no metastasis when implanted in syngeneic animals. The R3230AC mammary adenocarcinoma is a differentiated tumor of spontaneous origin which metastasizes in 20 to 50% of the animals inoculated. All 3 tumors are syngeneic to C. D. Fisher (F344) rats (Charles River Breeding Laboratories Inc., Wilmington, Mass.).

The 3 tumor cell lines were adapted to growth in tissue culture. Cells were cultured in 75-sq cm plastic flasks in RPMI Medium 1640 containing 10% calf serum, penicillin G (100 units/ml), and streptomycin (50 μg/ml). The cultures were grown at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Subculturing was done every second day. Early cell passages were used in all experiments. The cells were trypsinized, washed 3 times, and resuspended in RPMI Medium 1640 (medium and added components were obtained from Gibco, Burlington, Ontario, Canada) before inoculation into female F344 rats.

A tumor footpad model was used to follow lymphatic metastasis to the draining popliteal lymph node (5). A total of 106 cells in 100μl were inoculated s.c. into the right hind footpad via a 26-gauge needle. Primary tumor growth was measured in units of 0.1 ± 0.05 (S.D.) mm with a dial gauge caliper (Schellfaster; H. C. Kroeplin, Hessen, W. Germany). The left footpad was considered as normal and its width...
was subtracted from the width of the tumor-bearing footpad. Periodic palpation of the right popliteal lymph node was used to determine the presence of macrometastasis in the lymph node. At the termination of an experiment, the rats were again examined for macrometastasis in the popliteal lymph node and all body organs. The animals were also screened for micrometastasis by examining 5 to 8 histological sections of both the popliteal lymph node and lungs.

Serum Specimens. Each rat was bled from the tail vein on the day of tumor cell inoculation and then periodically thereafter. Approximately 1 ml of blood was taken each time. At the end of an experiment, larger blood samples were taken by cardiac puncture under anesthesia. The blood was clotted at 4° overnight, the clots were dislodged, and the samples were centrifuged at 1000 x g for 10 min. Each serum was dispensed in 50-μl aliquots in Eppendorf polypropylene microcentrifuge tubes (Brinkman Instruments, Inc., New York, N. Y.). All specimens were stored at −20°. An aliquot was used only once, immediately after thawing. Control sera were obtained from age-sex-matched normal rats.

Enzyme-conjugated Indicator Antibodies. Rabbit antiserum against the heavy and light chains of rat IgG and goat antiserum against the heavy chain of rat IgM were obtained from Pel-Freez Biologicals (Rogers, Ark.). Immunoglobulin fractions were prepared for both antisera by precipitation with 18% (w/v) Na2SO4. Anti-rat IgM immunoglobulin was further purified by chromatography on DEAE-Sepharose (Pharmacia Fine Chemicals; Dorval, Quebec, Canada) to yield purified IgG. HRP-conjugated IgG and IgM were obtained from Sigma Chemical Co. (St. Louis, Mo.). Conjugation of HRP to the immunoglobulin and IgG fractions of anti-rat IgM and IgG antiserum, respectively, was performed according to the method of Nakane and Kawaoi (12), except for omission of the fluorodinitrobenzene blocking and molecular sieve chromatography steps. The anti-rat IgG and IgM enzyme conjugates were used at a dilution (1:2500 in both cases) which gave an EIA A492 reading of 2.5 following incubation with solid-phase adsorbed rat IgG (Sigma) and 18% Na2SO4-precipitable rat immunoglobulin, respectively. The antirat IgM enzyme conjugate did not bind to solid-phase adsorbed rat IgM at dilutions ≥1:50.

Assay Buffers. The following buffers were used in both the C1q IC EIA and the RF EIA. Adsorption buffer consisted of 0.1 M Tris-HCl, pH 8.2, containing 0.1 M NaCl and 10-4 M merthiolate. Specimen buffer consisted of 0.01 M Tris-HCl, pH 8.2, containing 0.04 M NaCl, 0.5% (w/v) bovine serum albumin, 1.5% (v/v) Tween 20, and 10-4 M merthiolate. These 2 buffers were prepared in bulk and stored at 4°. Indicator buffer consisted of RPMI Medium 1640 containing 10% (v/v) C1q-depleted porcine serum, 2% (w/v) Tween 20, and 10-4 M merthiolate. Indicator buffer was prepared as required. The supernatant recovered after the first of 2 precipitations used in porcine C1q isolation (22) was used as the C1q-depleted porcine serum. It was stored at −20° and used after one thawing only.

Rat ΔlgG. Rat ΔlgG was initially used as an IC-positive sample in establishing the solid-phase C1q EIA for rat ICs. Subsequently, ΔlgG was included in all assays as a comparative reference. Rat IgG was dissolved in adsorption buffer (2 mg/ml) and heated at 63° for 12 min followed by cooling to room temperature. New ΔlgG was prepared prior to each assay.

C1q IC EIA. A solid-phase porcine C1q IC EIA originally developed for human IC detection (22) was adapted to detect rat ICs. Porcine C1q, 5 μg in 200 μl of adsorption buffer, was added to wells of flat-bottomed EIA plates (Dynatech Laboratories, Inc., Alexandria, Va.). Using more C1q per well did not significantly increase subsequent binding of rat ΔlgG, whereas using less caused ΔlgG binding to drop. C1q adsorption proceeded for 2.5 hr at room temperature, after which unadsorbed C1q was dumped from the wells. After each plate was washed 4 times with adsorption buffer, 200 μl of specimen buffer were added to each well.

Serum specimens were not heat inactivated in order to avoid formation of aggregated immunoglobulins and/or dissociation of heat-labile ICs (7). However, after serum specimens were thawed and centrifuged at 1000 x g for 10 min, they were treated with a chelating buffer to remove Ca2+ and thus inactivate endogenous complement (23). Each serum was diluted 1:3 with 0.2 M EDTA, pH 5.5, and incubated at 37° for 30 min. Seven-μl aliquots were then added in triplicate to EIA plate wells giving a final serum dilution of 1:90. After a 2-hr incubation at 37°, the plates were emptied and washed 4 times with distilled water. Anti-rat IgG enzyme conjugate in 200 μl of indicator buffer was then added to each well. After a 1-hr incubation at 37°, the plates were emptied and again washed 4 times with distilled water. Two hundred μl of enzyme substrate solution [0.1 M sodium citrate-phosphate, pH 5.5, containing freshly dissolved o-phenylenediamine (3 mg/ml) and 0.02% (v/v) H2O2] were added to each well. After 30 min in the dark at room temperature, the enzyme reactions were stopped by adding 100 μl of 2 N HCl to each well. A492 values were then read in a Gilford Instrument Model 250 spectrophotometer fitted with a Model 2443A rapid sampler and a Model 4019 thermal printer.

Rat ΔlgG was included in each assay as a positive reference. Several concentrations were assayed for in the presence of 1:90 diluted normal, pooled rat serum. A492 values obtained from wells containing only specimen buffer during the first incubation were used to correct for nonspecific binding of enzyme-conjugated indicator antibodies to the solid-phase C1q. Using an arbitrary corrected A492 value of 0.2 as a cutoff gave a sensitivity threshold for the assay of approximately 250 ng rat ΔlgG per ml. This is comparable to the 160- to 320-ng/ml amount of heat-aggregated human IgG that can be detected in the presence of 1% normal human serum with this assay (22). Testing of pre-tumor rat sera for base-line IC binding levels gave a mean A492 value of 0.070 ± 0.010. All sera collected from a given tumor-bearing rat were always tested in the same assay. Assay reproducibility was high, since repeat testing of different aliquots from each serum specimen series yielded results within 5% of the first analysis.

Serum Fractionation. Molecular sieve chromatography of 45-μl serum aliquots was carried out on an 8.2-ml (7.5-cm-high) column of Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, Calif.). Column buffer consisted of 0.1 M Tris-HCl, pH 8.2, containing 0.1 M NaCl. A flow rate of 4.2 ml/hr was used, and chromatography was done at room temperature. Fractions containing approximately 240 μl were collected, and 7-μl samples were added in triplicate to wells of an EIA plate containing adsorbed C1q and 200 μl of specimen buffer. ICs binding to the C1q were then detected by the EIA procedure already described. Elution profiles of blue dextran and rat IgM and IgG immunoglobulins were used for column calibration. Protein in each of the collected fractions was adsorbed to wells of an EIA plate, and rat IgM and IgG were then detected with HRP-conjugated anti-rat IgM and IgG antibodies, respectively.

RF EIA. Purified rat IgG was diluted to 25 μg/ml with phosphate-buffered saline (pH 7.3), and 200-μl aliquots were added to wells of EIA plates. After an overnight incubation at room temperature, unadsorbed IgG was removed, and the plates were washed 4 times with distilled water. Each rat serum was diluted 1:90 in specimen buffer, and 200 μl were added in triplicate. After a 2-hr incubation at room temperature, the samples were removed, and the plates were washed 4 times. Bound RF was then detected using HRP-conjugated anti-rat IgM antibodies. A492 values obtained for wells containing no rat serum were used to correct for background binding in the RF EIA.

Statistics. Formation of ICs in rats bearing metastatic and nonmetastatic R3230AC tumors were compared using the Biomedical Computer Programs P-series (9). Analysis of variance and covariance of a repeated measures design (one grouping and one trial factor) was used. The Mann-Whitney U test was used to compare RF levels in rat sera collected at various times after tumor inoculation.

RESULTS

Expression of IC Levels

How to express results obtained with any IC assay is a
problem due to the lack of a universally accepted standard. Many laboratories report IC levels in terms of \( \mu \text{g} \Delta \text{IgG} \) per ml. However, because different procedures are used in preparing \( \Delta \text{IgG} \), it is difficult to directly compare results from one report to another. Due to the longitudinal nature of our experiments, we present our IC data in terms of increases in \( A_{492} \) values for sera collected after tumor inoculation. The pre-tumor serum specimen thus forms the base line or zero IC level for each animal. In an attempt to provide a comparison for those laboratories using \( \Delta \text{IgG} \) equivalents to express IC levels, we also indicate the approximate \( \mu \text{g} \Delta \text{IgG} \) per ml equivalent for the maximum or plateau IC levels observed.

Circulating ICs in Tumor-bearing Rats

**13762 Tumor.** Rats inoculated in the footpad with 13762 tumor cells survive for 30 to 40 days with death invariably due to extensive metastasis. A total of 7 rats bearing 13762 tumors were analyzed. Palpable metastasis in the draining lymph node was found in 4 animals as early as Day 13. By Day 23, all 7 rats had palpable metastasis which, by the end of the experiment (Day 34), ranged from 0.25 to 2.0 cm in diameter. Primary tumor growth and formation of ICs in these animals both increased in a linear fashion (Chart 1). Only low levels of ICs were detected, reaching a maximum mean \( A_{492} \) of 0.050 (equivalent to approximately 10 \( \mu \text{g} \Delta \text{IgG} \) per ml).

**DMBA8 Tumor.** A total of 12 rats were inoculated with DMBA8 tumor cells. The rats were monitored over a period of 56 days and then sacrificed due to the large primary tumor burden. There was no evidence of either macro- or micrometastasis in these animals. IC levels in DMBA8 tumor-bearing animals (Chart 2) reached a maximum mean \( A_{492} \) of 0.065 at the end of the experiment (equivalent to approximately 12 \( \mu \text{g} \Delta \text{IgG} \) per ml). These IC levels are similar to those found in animals bearing the 13762 tumor (Chart 1). However, IC formation in DMBA8 tumor-bearing animals essentially reached maximum levels (by Day 34) prior to the most rapid growth of the primary tumor.

**R3230AC Tumor.** The 18 rats inoculated with R3230AC tumor cells were sacrificed at Day 100 due to large primary tumor burden. The animals were divided into 3 groups: rats with no metastasis (9 animals); micrometastatic tumors (5 animals); and macrometastatic tumors (4 animals). No difference was found in primary tumor growth among these 3 groups (Chart 3). Palpable metastasis was first detected in 2 animals on Day 46 and in another 2 animals on Day 81. Two of these 4 animals also developed lung metastasis, but no metastases were detected in other body organs. Histology of the secondary tumor in the popliteal lymph node from animals with
both macro- and micrometastasis was identical to histology of the primary footpad tumors.

IC levels found in R3230AC tumor-bearing rats (Chart 4) were much higher than IC levels found in animals bearing 13762 (Chart 1) or DMBA8 tumors (Chart 2). Animals bearing nonmetastatic R3230AC tumors developed the highest levels of ICs (Chart 4), and from Day 24 onward, IC levels were significantly higher than IC levels found in animals developing either macro- or micrometastasis ($p < 0.03$). IC levels in animals developing macro-R3230AC tumor metastasis did not differ significantly from those in animals developing micrometastasis ($p > 0.5$). IC levels in sera from rats bearing nonmetastatic tumors reached a maximum mean $A_{492}$ of 0.299 (equivalent to approximately 55 µg AlgG per ml) on Day 65. IC levels in animals developing macro- or micro-R3230AC tumor metastasis reached maximum mean $A_{492}$ values of 0.137 (Day 81) and 0.135 (Day 65), respectively (equivalent to approximately 25 µg AlgG per ml). In each of the 3 groups, IC formation essentially reached maximum levels by Day 46. In contrast, primary tumor size increased more rapidly after Day 54.

Size of ICs

Sera from several R3230AC tumor-bearing rats were fractionated by molecular sieve chromatography in order to determine sizes of ICs present. Pre-tumor sera from the same animals were also fractionated to obtain a background IC size profile for comparison. The IC size profile for a rat bearing a terminal, nonmetastatic R3230AC tumor is shown in Chart 5. Three major IC peaks were found. The majority of the ICs had a molecular weight of less than $10^6$. Terminal sera from rats bearing either macro- or micrometastatic R3230AC tumors had similar IC size profiles.

Rheumatoid Factor

All rat sera examined for ICs were also tested for RF. Pre-tumor sera gave a median $A_{492}$ value of 0.193 in the RF EIA (Chart 6). RF levels in sera from animals bearing 13762 and DMBA8 tumors did not differ from normal (only data for terminal sera are shown in Chart 6). RF levels in sera from R3230AC tumor-bearing rats were significantly elevated starting at Day 65 (Chart 6). Comparison of RF levels on Days 65, 81, and 100 (terminal) sera to RF levels in pre-tumor sera yielded $p$ values of $<0.01$, $<0.005$, and $<0.001$, respectively. RF levels in animals bearing R3230AC tumors with macrometastasis, with micrometastasis, or with no metastasis did not differ significantly from each other. Significant elevated RF synthesis in animals bearing R3230AC tumors began only approximately 3 weeks after IC formation had reached plateau values (compare Charts 4 and 6).

DISCUSSION

The first conclusion to be drawn from this study is that, for the 3 different tumor cell lines studied, formation of ICs is not clearly related to the ability of the tumor cells to metastasize. This is based on the finding that rats bearing the low-frequency-metastatic R3230AC tumor developed high levels of ICs, whereas rats bearing highly metastatic 13762 or nonmetastatic DMBA8 tumors developed much lower IC levels. The second conclusion is that detectable IC levels need not directly relate to tumor load, since rats bearing metastatic R3230AC tumors...
had significantly lower IC levels than did those animals bearing nonmetastatic tumors. As well, in rats bearing either DMBA or R3230AC tumors, plateau IC formation occurred prior to most rapid tumor growth. Nonetheless, the fact that rats bearing metastatic R3230AC tumors had IC levels significantly different (even though lower) than did rats bearing nonmetastatic tumors still suggests that the host's immune response (one consequence of which may be IC formation) may determine whether metastasis occurs.

Formation of ICs in tumor-bearing animals depends on the ability of the tumor cells to shed cell surface antigens and on the host's immune response towards these antigens. If either component is lacking, then circulating ICs will not be formed. As well, modulation in either component logically could lead to varying levels of circulating ICs. Rapidly growing and metastasizing tumors are known to have a high metabolic turnover and shedding of surface antigens (11). Thus, for the highly metastatic 13762 tumors, large amounts of cell surface antigen released could either tolerate the immune response or lead to tumor antigen excess so that high levels of ICs do not form. On the other hand, the nonmetastatic DMBA tumor may not be capable of shedding surface antigens, again leading to low level IC formation. In support of this, we have detected large amounts and essentially normal amounts of sialoglycoproteins in sera of animals bearing 13762 tumors and DMBA tumors, respectively.

In the R3230AC tumor system, there appears to be a balance between antigen, release and antibody production resulting in high levels of circulating ICs, i.e., an "inbetween" model of the other 2 tumor systems. Several factors could account for the fact that rats bearing metastatic R3230AC tumors have significantly lower levels of ICs than do animals bearing nonmetastatic tumors. An anergic immune state could be induced by excess surface antigen release by the metastasizing tumor such that lower levels of ICs are formed. Alternatively, the host's humoral response to tumor cell surface antigens may be suppressed by other types of blocking factors. The lower level of circulating ICs found in animals bearing metastatic R3230AC tumors could also be due to more rapid clearance of ICs in animals developing metastasis.

Clearance of antigens from the circulation after being bound to antibody is dependent primarily upon the ratio of antigen to antibody in the circulation (21). Large (greater than 11S) ICs are rapidly cleared from the circulation by the mononuclear phagocytic system (2). Smaller ICs are not cleared as easily and can persist in the circulation for longer periods (17). Molecular sieve chromatography fractionation of ICs in sera from animals bearing the R3230AC mammary adenocarcinoma revealed the ICs to be small to intermediate in size. Other investigations of size profiles of ICs in sera from tumor-bearing animals have also shown that the ICs are predominantly of small to intermediate size (7).

RF levels in sera from rats bearing the 3 types of mammary adenocarcinoma tumors were ascertained for 2 reasons: (a) to determine if RF was interfering with detection of ICs by the C1q immunoassay; (b) to determine if RF synthesis correlated with formation of ICs. No abnormal RF synthesis was found in animals bearing 13762 and DMBA tumors. This finding suggests RF does not interfere with detection of rat ICs by the C1q

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D. B. S. Hoon et al.

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