Comparison of Antibody Isotypes in Sera and Circulating Immune Complexes during Tumor Growth and Metastasis of Three Tumor Models in Mice^1

Thomas L. McDonald,2 Margaret Collins, and James E. Talmadge

University of Nebraska Medical Center, Department of Medical Microbiology, Omaha, Nebraska 68105 [T. L. M.], and Preclinical Screening Laboratory, NCI-Frederick Cancer Research Facility, Frederick, Maryland 21701 [M. C., J. E. T.]

ABSTRACT

The sera and circulating immune complexes (CICs) from mice bearing either UV-2237 mm, K-1735 M2, or B16-BL6 were analyzed for antibody isotype distribution during primary tumor growth and spontaneous metastasis. These studies clearly demonstrate that elevated CIC concentrations parallel the initial stages of primary tumor growth; however, the CICs, as well as serum concentration of all immunoglobulin G isotypes, rapidly decrease to near normal levels in the presence of heavy tumor burden. Following resection of the primary footpad tumor, increases in CIC levels paralleled outgrowth of metastasis; however, the antibody content of the CICs was isotypically restricted to immunoglobulin G2b and immunoglobulin G3 regardless of the tumor type. The immunoglobulin G isotype content of the CICs did not correspond to elevations in the serum concentrations of each respective isotype during either primary nor metastatic tumor growth. This serial study indicates that the use of non-antigen-specific assays for CICs without regard to tumor burden or possible antibody isotypic restriction may be misleading, and that CIC levels can indeed correlate with metastatic tumor burden.

INTRODUCTION

Metastasis is a complex multifaceted process that is dependent upon both intrinsic tumor cell properties and host factors (7). One such tumor-host interaction is the production of antibodies with the subsequent formation of CICs.3 The serum elevation of CICs have been identified in a wide spectrum of disease states, including autoimmune, infectious, and parasitic diseases (reviewed in Ref. 30), in addition to neoplastic disorders (25, 31). Recent investigations have suggested that the level of CICs may have some value as a prognostic indication of tumor recurrence and extent of tumor burden (13, 19, 22); however, these studies have been controversial (2, 21). The controversy stems largely from the observation that the CIC levels are not associated primarily with tumor burden, but may be secondary to opportunistic infections common to the immunocompromised patient. In addition, the assessment of the significance of CIC levels may be limited by the heterogeneity of the CICs, the tests used for CIC detection, and the timing of sample acquisitions.

The present study undertook the investigation of serial CIC levels during primary growth and spontaneous metastasis of several virus-free tumors in specific pathogen-free animals. Additionally, we examined the IgG antibody subclasses associated with the CICs, as well as their respective serum concentrations, in order to evaluate whether isotypic restriction occurred during the process of metastasis.

We report that within these controlled experimental conditions, the level of CICs closely parallels the development of metastatic disease; however, CIC levels may not reflect primary tumor growth. Furthermore, the antibody subclass that comprise the CICs appear to be isotypically restricted to IgG3 and IgG2b, regardless of the tumor model or host strain.

MATERIALS AND METHODS

Animals. Specific-pathogen-free male C57BL/6N-H-2^b mice and C3H/HeN-H-2^b (murine tumor virus-negative) mice, 4 weeks of age, were obtained from the animal production areas of NCI-Frederick Cancer Research Facility, and were used as an age- and sex-matched colony. Tumors. These studies utilized a variant of the UV radiation-induced fibrosarcoma, UV-2237, syngeneic to the C3H-H-2^b mouse (3). The variant, UV-2237 mm, is a cell line derived from 5 spontaneous metastases of the parent UV-2237 tumor, and is highly metastatic (24). The second tumor used was a variant of K-1735, a UV radiation-induced, croton oil-promoted malignant melanoma, syngeneic to the C3H mouse (14). This variant, K-1735 M2, was obtained from a single, well-isolated spontaneous metastasis, and is highly metastatic.

The other cell line used in these studies is a variant of the malignant melanoma B16, which was selected 6 times in vitro for its ability to invade through the bladder epithelium (6). This tumor, B16-BL6, is highly invasive and spontaneously metastatic in syngeneic C57BL/6N mice.

These cell lines were maintained as monolayers in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a 2-fold vitamin solution. All cell lines were free of Mycoplasma and the following murine viruses: reovirus type 3, pneumonia virus of mice, K virus, Theiler's virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus.

Experimental Design. A single-cell suspension of tumor cells (5 x 10^6) in 0.05 ml of Ca^2+-Mg^2+ Hanks' balanced salt solution were inoculated into the posterior footpads of 6-week-old syngeneic mice. The tumors were measured twice a week, and when they reached a diameter of 1.0 cm, the tumor-bearing leg was resected at midfemur to include the popliteal lymph node. Groups of 5 mice were bled when the footpad tumors were 0.25, 0.5, 0.75, and 1.0 cm in diameter; an additional blood sample was taken at 1.5-cm-tumor diameter for K-1735 M2 and UV-

Received January 27, 1984; accepted July 16, 1984.

NOVEMBER 1984

4933

[CANCER RESEARCH 44, 4933-4937, November 1984]
2237 mm. Cohorts of mice were bled at 1, 7, 14, 21, and 28 days following resection of the primary tumor; an additional blood sample was taken from UV-2237 mm and K-1735 M2 tumor-bearing mice at Day 35. Mice bearing B16-BL6 were not bled at Day 35, since the animals were moribund by that time. The lungs from all animals were retained and fixed in Bouin's fixative to provide a gross estimate of metastatic tumor burden.

Since one of the major objectives of this research was to determine the relationship between CIC levels and tumor progression, the size of the primary tumor was used to determine when individual animals were bled to contribute to a serum pool, and not the time elapsed following tumor cell inoculation. In this way, any individual variation in the rate of primary tumor growth would be normalized.

CIC Isolation. Immune complexes were isolated from pooled sera of tumor-bearing and normal mice by equine RHC (15), as described by McDonald et al. (17), with one exception; 100 μl of sampled pooled mouse sera were added to 400 μl of RHC (1.0 mg protein/ml in 50 mM Tris-HCl-0.5 M NaCl, pH 8.2) instead of equal volumes of serum to reagent.

Quantification of IgG Subclasses. The RHC reagent and coprecipitated CICs were dissolved at 56°C for 15 min in phosphate-buffered saline containing 0.1% sodium dodecyl sulfate, pH 7.2. Quantitation of antibody content of the CICs and sera, as well as identification of antibody subclasses, was done by radial immunodiffusion. The appropriate antisera (Meloy Laboratories, Inc., Springfield, VA) were added to 56°C agarose (1% w/v of barbital buffer, pH 8.6), and 10.0-μl samples were added to precut wells (3.3 mm) for overnight diffusion. Standards of each IgG subclass (Research Products International, Elk Grove Village, IL) at 100, 50, 25, 10, and 5 μg/ml were used for quantification. Because of the limited amount of serum available for assay, data points for subclass analysis of IgG-containing CICs and sera IgG subclass concentrations are represented by an average of duplicate samples run at 2 different times. The sensitivity levels for IgG1, IgG2a, IgG2b, and IgG3 were 5.0, 2.0, and 5 μg/ml, respectively. The coefficient of variation for interexperimental variation of duplicate samples was 4.6%. Intraexperimental variation of RHC isolation of CICs was 2.0%, and was determined on duplicate samples run at different times.

Radial immunodiffusion was also used to assure that the reactivity of the IgG subclass-specific antisera used in these experiments was monospecific. All 4 IgG subclass standards were diffused in each agarose gel containing one anti-IgG isotype antisera. No cross-reactivity of these reagents was detected in our assay system.

RESULTS

The data in Chart 1 represent the concentrations of CICs detected in samples of pooled sera collected from mice bearing a primary tumor of uniform size, or serially, following tumor resection and growth of the metastatic disease. Quantitation of the RHC-coprecipitated CIC was by radial immunodiffusion using anti-mouse IgG, H- and L-chain-specific antisera, which allowed for an estimation of the total serum concentration of CIC with each of the tumor models, regardless of the subclass of IgG present. Background levels of CICs that are inherent to the test are represented by 28-day sera from mice that were non-tumor-bearing animals (designated by N), but did undergo the same surgical resection procedure as tumor-bearing animals. The data clearly show that CICs are detectable in the sera of tumor-bearing animals during primary and metastatic tumor growth. The relative concentrations of CICs during primary tumor growth appeared to depend on the type of tumor and when the sample was taken. The highest CIC levels were consistently found during early tumor growth with the more immunogenic tumor, UV-2237 mm, and to a lesser extent, K-1735 M2 and B16-BL6. With both mouse models and all tumor types tested, CIC levels decreased when the primary tumor burden increased from 0.75 cm. With one of the tumors (K-1735 M2), this decrease was to levels near or below the background concentrations of CICs detected in non-tumor-bearing animals. One week following resection of the primary tumors, elevated levels of CICs were again detected in the sera of mice bearing microscopically detectable spontaneous metastasis. With the exception of K-1735 M2, the CIC levels increased rapidly during progressive metastatic tumor growth until termination of the experiment.

The humoral immune response, as reflected by the increase in serum IgG subclasses, to the primary and metastatic growth of the UV-induced tumors UV-2237 mm and K-1735 M2, are similar, as shown in Charts 2A and 3A, respectively. The subclass IgG1 clearly makes up the greatest percentage of IgG antibody produced in response to the moderately immunogenic UV-2237 mm tumor, with an approximate 9-fold increase observed during primary and metastatic tumor growth (Chart 2A). Serum concentrations of IgG3 and IgG2a subclasses were elevated in a pattern similar to that of IgG1; however, they were not significantly different than control values. The serum concentration of IgG2b in both UV-2237 mm and K-1735 M2 tumor-bearing animals did not increase above the normal values of C3H mice during primary or metastatic tumor growth. The pattern of increased serum IgG antibody subclasses during primary and metastatic tumor growth of the less immunogenic K-1735 M2 (Chart 3A) was identical to that of UV-2237 mm (Chart 2A), although the concentrations of each subclass at any one time were generally lower for K-1735 M2.

Analysis of these sera for CIC content did not reflect the similarity previously observed with the serum isotype response of C3H mice to these 2 tumors. With the UV-2237 mm (Chart 2B), CICs containing each of the 4 IgG isotypes were detected during primary and metastatic tumor growth. IgG2b was the predominant IgG isotype observed in the CIC, even though the serum concentrations of this IgG subclass did not increase above
The persistence of IgG3 containing CICs late in the metastasis of UV-2237 mm is not reflected by a continual increase of IgG3 antibody in the sera of these animals.

With the less immunogenic K-1735 M2 tumor, only CICs containing IgG3 were detected, either during primary tumor growth or during metastasis (Chart 3B). This was an intriguing observation, since the pattern of sera IgG subclass response to this tumor, both during primary growth and metastasis, was the same as for the UV-2237 mm tumor. It appears that the more antigenic UV-2237 mm tumor stimulates higher serum antibody and CIC titers than does the less immunogenic K-1735 M2 tumor. Moreover, there appears to be an antibody isotype shift to CICs containing predominantly IgG3 and IgG2b during metastasis.

The primary tumor burden seems to exert a regulatory effect on the serum antibody concentration (in particular, IgG1) and on levels of CICs with both UV-induced tumors tested (Charts 2 and 3). This is first observed after the primary tumor growth reaches approximately 0.75 cm in diameter and continues until resection of the primary tumor. Following resection, the increase of antibody subclasses in the sera is identical to the pattern observed during primary tumor growth, with elevated levels occurring by 1 week for IgG1, IgG2a, and IgG3.

In order to determine whether these observations were unique to UV-induced tumors in C3H mice or were characteristics shared with other metastatic tumor models, the experiments were repeated several times with the B16-BL6 tumor models in syngeneic C57BL/6N mice. The serum IgG subclass response and the CIC concentration of C57BL/6N mice during primary and metastatic tumor growth of the highly metastatic, highly invasive B16-BL6 tumor (Chart 4), was similar to that which was observed in C3H mice bearing UV-2237 mm and K-1735 M2 tumors. Serum concentration of IgG1, IgG2a, and IgG3 antibody subclasses increased during the early stages of primary tumor growth; however, as seen with all other tumor systems in this study,
there was a marked decrease in serum concentration of IgG1 when the tumor reached 0.75 cm in diameter (Chart 4A). Although not as evident, serum concentration of the other isotypes also decreased in a similar pattern during this time. CICs containing IgG3 and IgG2b were the only CICs detected during both the primary and metastatic tumor growth (Chart 4B). As in the case of UV-2237 mm metastasis (Chart 3B), sera from mice with B16-BL6 metastases had high levels of IgG3 and IgG2b containing CICs at the termination of the experiments. The serial increase in CIC concentration paralleled increased metastatic tumor burden.

Although the primary emphasis of this study was directed toward the evaluation of IgG isotypes in the CICs of mice bearing primary and metastatic tumors, we also analyzed all sera and CICs for the presence of IgM antibodies. In all cases, regardless of the tumor system or strain of mouse, the serum concentrations of IgM did not increase above normal control values, and IgM-containing CICs were never detected during primary tumor growth or their corresponding metastases.

**DISCUSSION**

There are 3 major problems that have hampered previous efforts designed to correlate CIC levels and tumor growth, both clinically and in experimental tumor models. First, non-antigen-specific assay systems used for CIC detection may not detect all types of antibody-containing CICs. This is an important consideration in data evaluation, since it was recently shown (29) that, during progressive tumor growth of Hepatoma D23, there appeared to be a shift from complement-fixing antibodies contained in the CICs to non-complement-fixing antibodies. We have attempted to minimize this problem by using a reagent, equine RHC, that has previously been shown to coprecipitate all subclasses of antibody containing CICs from either mouse or rat serum (17, 26). Secondly, the various tumor cell lines may be contaminated with viruses, or infections may occur at the site of inoculation that could result in increased levels of CICs unrelated to tumor growth. This is of primary clinical importance, since some tumor-bearing patients have evidence of sepsis at the time of diagnosis. In addition, cancer patients undergoing immunosuppressive therapy may acquire a variety of bacterial, viral, or fungal infections that could contribute to the CIC pool detected by non-antigen-specific assays. In our experiments, all animals used were specific pathogen free, and were maintained in holding facilities that were specific pathogen free. Also, all tumor cell lines used in these experiments were screened for viral and bacterial contamination prior to inoculation. These precautions essentially eliminated the possibility that CIC measurements were not tumor associated. A third problem is that previous studies of CIC measurements and tumor growth may not reflect the heterogeneous nature of tumors, and such data may be unique to that system only. Our approach to this criticism was to use 2 UV-induced tumor cell lines syngeneic to C3H mice, and a variant of a spontaneous malignant melanoma, syngeneic to the C57BL/6N mice. As noted earlier in this paper, in each of these models, metastasis occurs spontaneously from the primary tumor, with a 90 to 100% incidence. The use of these spontaneously metastizing tumor cell lines in conjunction with IgG subclass antibody analysis of CICs gave us a unique opportunity to not only determine CIC occurrence during tumor growth, but to evaluate whether quantitative differences in CICs correlate with primary tumor growth or tumor origin, and whether antibody isotype restrictions occur.

In general, our results show that CICs occur at elevated levels in the sera of mice during the early stages of primary tumor growth, irrespective of the tumor type or strain of mouse tested. However, these elevated levels of CICs were not representative of tumor burden immediately prior to resection of the primary tumor; for example, with K-1735 M2, the tumor size increased from 0.5 to 1.5 cm; however, the CIC levels decreased below normal concentrations. These results are in agreement with those reported by Jennette and Feldman (11), with progressive Moloney sarcoma virus-transformed cells in BN rats. These authors reported that high levels of CICs occurred at an early stage in tumor growth, decreased just before the terminal phase of tumor progression, and continued to decline, despite massive tumor loads before death. Hoffken et al. (9) reported similar results with rats bearing chemically induced tumors growing at s.c. sites or in the peritoneal cavity. In these instances, C1q-binding material in the serum reached a maximum level at nominal tumor burden, and decreased despite tumor progression. These CIC levels eventually fell into subnormal ranges at terminal stages of tumor growth.

With the 3 tumor models we tested, the decrease in CIC levels despite increasing primary tumor burden could be due to the different proportions of antigens or antibodies in the serum during the course of tumor growth. It would appear likely that the CICs contain tumor-related material, rather than extraneous material from the inoculum or infectious agents, since the CICs were detected throughout most of the primary and metastatic tumor growth, and specific-pathogen-free animals were used. Changes in the rates of antigen shedding from the tumor could alter the nature of the CIC such that they would be more or less soluble, and possibly more or less reactive with the CIC-isolating reagent. In addition, rates of antibody adsorption by tumors, CIC clearance by the reticuloendothelial system, and qualitative and quantitative changes in neoplastic tissue during the time course of tumor growth, could explain the CIC profiles reported here.

The apparent decrease in serum antibody, in particular IgG1, in mice with extreme primary tumor burden could be due to the inhibitory effects of the CIC, as previously suggested by Hellström and Hellström (8), Baldwin et al. (1), McDonald (16), Rodrick et al. (20), and by many others (reviewed in Ref. 4); however, another possible explanation is that tumor antigens, shed during the primary growth, are immunoregulatory, as shown previously by Kalish and Brody (12) with the B16 melanoma. These authors suggested that tumor-facilitating factors shed from the surface of in vitro cultures of B16 melanoma were enhancing for tumor growth when injected into C57BL/6J mice prior to tumor inoculation. Similarly, tumor-suppressive factors not associated with CICs have been reported in other systems (5, 28). Our data are insufficient to determine whether the apparent decrease in IgG antibody and CICs that occurred during increasing primary tumor burden is due to immunoregulatory properties of tumor products, possibly IgG1 antibody, or due to other factors. However, our results from these serial studies may explain the inconsistency in data from various human studies that have attempted to use non-antigen-specific CIC assays as a diagnostic or prognostic indicator of tumor growth (1). Our results show that the time of the sample acquisition, with respect to tumor burden, appears to an important consideration in detecting CICs.

With respect to subclass analysis of the CICs during primary
tumor growth and their corresponding metastases, our data show that the most predominant subclasses of IgG detected in the CICs were IgG3 and IgG2b. Although the relative concentrations of CICs containing these 2 subclasses of antibody were higher during the later stages of metastasis than during primary tumor growth, there was no obvious shift in isotype patterns. The subclasses of IgG2a and IgG1 were only detected in the CICs obtained from the more antigenic UV-2237 mm tumor during both primary growth and metastasis; however, they had decreased to near normal (IgG2a) or undetectable (IgG1) levels by 4 weeks following resection of the primary tumor. The differences observed in the IgG subclasses of the CICs generated in different tumor systems may reflect the heterologous nature of the antigens shed from the tumor surface. It has been shown, for example, that small changes in membrane fluidity were responsible for altered antigen shedding by tumor cells that led to a different serological response (23). Also, a marked decrease in IgG2a containing CICs was shown to occur when an established murine neuroblastoma tumor cell was used as inoculum, as compared to the neuroblastoma tumor, and indicated a shift in antigenic expression caused by subculturing primary tumor (29).

Since many dynamic factors contribute to the rate and quality of tumor antigen generated, the response of the immune system, and the clearance kinetics of CICs, it is beyond the scope of this report to project the significance of the persistence of elevated levels of IgG2b and IgG3 containing CICs in UV-induced tumors and in B16 malignant melanoma. It is obvious that using this approach, analysis of the CICs for antigen content will be required to determine whether or not primary tumor growth and their corresponding metastases share antigen, as suggested recently by Thist lethweithe et al. (27) with UV-K-1735, and Papsidero et al. (18) with monoclonal antibody to breast cancer-related CICs.

We are currently analyzing the CICs isolated from the sera of C57BL/6N mice bearing B16 melanoma by polyacrylamide gel electrophoresis, in an attempt to identify common (or unique) tumor-associated antigens that may occur during primary and metastatic growth. Using this approach, we may be able to utilize the immune response of the host in order to identify antigens associated with tumor growth in vivo, as opposed to in vitro antigenic expression.

ACKNOWLEDGMENTS

The authors acknowledge the excellent technical work of Annika Weber.

REFERENCES

Comparison of Antibody Isotypes in Sera and Circulating Immune Complexes during Tumor Growth and Metastasis of Three Tumor Models in Mice

Thomas L. McDonald, Margaret Collins and James E. Talmadge

Cancer Res 1984;44:4933-4937.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/44/11/4933

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.