Cell Cycle Dependency of Human Sarcoma-associated Tumor Antigen Expression

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SUMMARY

An analysis of cell cycle-dependent expression of tumor-associated antigen was performed on a human neurosarcoma cell line (T2 cells). The expression of sarcoma-associated tumor antigen on T2 cells was detected using test sera obtained from sarcoma patients; control sera were from patients with nonsarcoma neoplasias and from normal donors. Results indicate a progressive increase in the antigenic expression beginning in late mitosis and early G1, with maximum expression in mid-G1. Antigenic expression declines to minimum levels in S and G2-phase. Mechanisms responsible for this cycle-dependent fluctuation are presently unknown.

INTRODUCTION

Unique tumor cell components (i.e., tumor-associated antigens) capable of eliciting an immunological response from the tumor-bearing host have been detected in a variety of human and animal systems (12, 13, 15, 16, 19, 21, 23, 25, 29, 30). There is evidence suggesting that the appearance of such tumor antigens may be related to the transit of malignant cells through the proliferative cycle (5, 17, 24, 34). Cikes (5) reported maximum expression of surface antigen during G1 phase for murine lymphoma cells (YAC). Cikes and Friberg (6) reported maximum expression of H-2- and murine leukemia virus-determined surface antigens in G1 for another mouse lymphoma cell line (JLS-V9) (6). Stenman et al. (35) recently reported the occurrence of peak expression of the T-antigen gene(s) for SV40 and the T-antigen component in the G2-phase of an SV40-transformed human cell line. Similarly, Panem and Schauf (24) detected murine gs antigens at peak values during S and G2. In addition, several laboratories have investigated the significance of cell cycle-related events to the synthesis and/or expression of a wide variety of macromolecules (4, 8, 36, 38-40). However, biosynthetic events responsible for this cycle-dependent fluctuation are presently unknown.

The purpose of this paper is to describe cell cycle relatedness of SATA, demonstrated on an established human cell line derived from a neurogenic sarcoma. Previous studies had demonstrated the presence of a tumor antigen common to sarcomatous neoplasias, on the surface of the cultured cells (9, 18). SATA was detected by immunofluorescence and immunoperoxidase electron microscopy techniques, using sera from patients with various types of sarcomas. Several such sera were used in this study to reveal G1 phase as the stage of maximum SATA expression.

MATERIALS AND METHODS

Cell Cultures

The cell line (T2 cells) used in these studies was derived from a neurofibrosarcoma of a 42-year-old woman (41). T2 cells have been maintained as monolayer cultures for 9 years in Ham's F-10 medium supplemented by 20% fetal calf serum, vitamins, glutamine, and antibiotics. Cell kinetics parameters under these conditions are: generation time, 58 hr; G1 phase, 27.5 hr; S phase, 22 hr; G2 phase, 8.5 hr; and doubling time, 63 ± 3 hr. The time for mitosis calculated from the mitotic index is 1 hr. Cells are free of Mycoplasma contamination as routinely monitored by biochemical (1) and electron microscopic means.

Other human long-term tissue culture cell lines, which included lymphoma (42), melanoma (31), adenocarcinoma of colon (10), normal skin fibroblasts, and a cell suspension from peripheral nerve tissue obtained from human cadavers, were utilized for serum absorption procedures.

Synchronization Procedures

T2 cells were treated with 4 mM TdR for 48 hr to obtain synchronized S- and G2-phase cells. The degree of synchrony was monitored by the LI and the MI. Cells were pulse labeled at 2-hr intervals for 30 min with [3H]TdR, 1 μCi/ml (9.59 Ci/m mole). Autoradiographs were prepared by dipping cytocentrifuge preparation slides in Ilford K5 emulsion, diluted 1:1 with distilled water. The LI was evaluated by counting 500 cells (labeled cell, greater than 24 grains/nucleus) and the MI was evaluated by scoring 1000 to 2000 cells.

To obtain mitotic cells and cells in G1 phase, the selec-
tive detachment of mitotic cells technique (37) was employed. Cells grown in diphtheria toxin bottles (250 ml of medium) were first blocked with 4 mM TdR, as described above. After removal of the TdR block, the cells were allowed to progress synchronously through S and G2 phase. Mitotic cells were then detached, pooled, and collected on ice. The M1 was assessed and aliquots were plated into fresh medium, incubated at 37°, and allowed to progress into G1. The synchronous progression of T2 cells through G1 was monitored by pulse labeling at 2-hr intervals for 30 min with [3H]CdR and recording the LI and MI.

Serum Collection

Serum samples were obtained from patients with confirmed diagnosis of sarcoma. Individuals with a history of blood, platelets, or granulocyte transfusion, pregnancy, or any form of antitumor therapy were excluded. Control sera were collected from individuals with nonsarcomatous neoplasia including malignant melanoma, lymphoma, leukemia, multiple myeloma, epithelial tumors, and from apparently healthy blood bank donors. All sera were inactivated at 56° for 30 min and stored at -20°.

Immunofluorescent Screening of Sera

Membrane-bound Antigen. Test and control sera were screened for the presence of anti-SATA antibody by incubating 0.1-ml aliquots with 5.0 x 10^6 T2 cells for 30 min at 37°. At the end of the incubation period, the samples were centrifuged, the supernatant serum was discarded, and the pellet was washed 3 times in FTA buffer (Difco Laboratories, Detroit, Mich.). Cells were subsequently incubated with 0.1 ml of a 1:10 dilution of FITC-labeled goat anti-human immunoglobulin for 30 min at 37°. Samples were centrifuged, washed in FTA buffer, and resuspended in Sorenson's buffered glycerol at pH 8.2. Cells incubated with 0.9% NaCl solution were included as controls in each test to rule out nonspecific fluorescence. Aliquots of each sample were placed on slides designed for fluorescent methods (Clay Adams, Parsippany, N. J.) and examined for the presence of antibody to SATA on T2 cells by the indirect immunofluorescence technique. Each serum was previously tested by microimmunoelectrophoresis against normal human serum to establish monovalent specificity of the antibody activity.

Intracytoplasmic Antigen. The multiprint slide technique described by Drewinko et al. (9) was utilized to screen intracytoplasmic SATA. Briefly, T2 cells were allowed to progress to exponential growth on sterile, multiprint slides. Slides were washed and fixed in acetone. Twenty μl of test serum, control serum, or 0.9% NaCl solution were added to appropriate spots and incubated for 30 min at 37°. Cells were washed 3 times in FTA buffer and partially air dried. Twenty μl of FITC-labeled goat anti-human immunoglobulin were added to each spot and incubated for 30 min at 37°. Slides were then washed in FTA buffer, partially air dried, mounted with Sorenson's buffered glycerol, and examined as unknowns by 2 independent observers under UV microscopy. Scoring criteria were as described before (9).

Absorption Procedures. Positive sera were absorbed sequentially with human red blood cells (type AB, Rh positive), established lines of human malignant lymphoma, melanoma, adenocarcinoma of colon, skin fibroblasts, and a suspension of human peripheral nerve tissue to eliminate nonspecific, cross-reactive antibodies. Each sample was absorbed with 5.0 x 10^8 cells/ml of serum for 1 hr at 37° with gentle agitation. After each absorption sera were retested against T2 cells.

Immunoglobulin Class Determination. All sera that remained positive following the absorption procedures were tested for the immunoglobulin class of the antisarcoma antibody activity with monovalent FITC-labeled antisera (anti-human IgG, IgA, IgM; Behring Diagnostics, Woodbury, N. Y.) by the indirect immunofluorescence technique. Each antisera was previously tested by microimmunoelectrophoresis against normal human serum to establish monovalent specificity of the antibody activity.

Immunokinetic Studies. Cell cycle-dependent expression of SATA was analyzed on T2 cells synchronized as described above. Cells for S- and G2-phase samples were blocked with 4 mM TdR for 48 hr, followed by 2 washes and the addition of fresh medium. Samples were harvested and prepared for indirect immunofluorescence at regular intervals throughout S and G2, using sera with high titers of anti-SATA antibody activity. Controls consisted of cells incubated with normal serum and 0.9% NaCl solution. Cells for M- and G1-phase determinations were synchronized by excess TdR block and mitotic selection. An aliquot of mitotic cells obtained by this technique was processed immediately. The remainder of the cell suspension was plated into fresh medium and sampled throughout G1 at regular intervals. Test and control sera were identical to the above.

In all immunokinetic studies, monovalent FITC-labeled anti-human IgG antiserum was used, and the degree of synchrony was monitored by pulse labeling with [3H]CdR followed by autorigraphy.

RESULTS

Synchronization of T2 Cells. A 48-hr treatment with 4 mM TdR yielded 85% cells in S phase as assessed by the LI. This level was maintained for about 22 hr after which it steadily declined to values of 20%. The MI peaked at 5% at 30 hr. Mitotic cells harvested by selective detachment procedures yielded a MI of 91%. The MI rapidly fell to less than 0.5% upon incubation at 37°, while the LI was 0. These values were maintained for about 26 hr, at which time the LI sharply rose to 57% at 32 hr, indicating the arrival of cells at S phase.

Anti-SATA Antibodies. Sera from 94 individuals were examined for the presence of antibody to SATA on T2 cells (Table 1). Of 33 patients with sarcoma, 21 reacted with T2 cells yielding 25 to 60% fluorescent cells. All reacted with live cells and, in addition, 8 presented intracytoplasmic fluorescence. Five of the nonsarcoma neoplasia group reacted with live cells, while 3 such sera yielded only intracytoplasmic fluorescence. A single blood bank donor gave both membrane and intracytoplasmic fluorescence. All 30 (31%) initially positive sera were absorbed sequentially with...
various cell suspensions to eliminate nonspecific cross-reacting antibodies and were retested on T2 cells following each absorption (Chart 1). Of the 21 sera from the sarcoma group, 19 (90%) remained positive after an exhaustive absorption procedure with the various cell panels. All 8 sera from the nonsarcoma neoplasia group were eliminated after absorption with RBC, lymphoma cells, and adenocarcinoma cells. However, the serum from the single blood bank donor remained consistently positive (43% fluorescent cells) despite absorption with the entire battery of cell panels. Only membrane fluorescence was detected with those sera that remained positive following absorption (19 sarcoma and 1 blood bank donor).

In addition to these procedures, all positive sera were tested against red cell panels of known phenotypic characteristics including Rh-Hr, MNSs, Lewis, P, Kell, Duffy, Kidd, Lutheran, and sex-linked blood group systems. Sera also remained positive after absorption with a human colon adenocarcinoma cell line, known to produce substantial amounts of CEA (52 ng of CEA per 10^6 cells) as assayed by the method of Egan et al. (11).

Immunoglobulin class determinations performed on all positive sera with monospecific antihuman antibody demonstrated that the anti-SATA activity resided in the IgG class.

**Immunokinetic Studies.** Immunokinetic studies were performed on synchronized populations of T2 cells utilizing 4 positive sarcoma sera (identified as A, B, C, and D) and 2 negative sera as assayed by the above-indicated procedures. Additional controls consisted of cells incubated with 0.9% NaCl solution. All cells were incubated with monovalent FITC anti-human IgG. Asynchronous T2 cells had yielded 51, 57, 41, and 51% fluorescent cells when incubated with serum A, B, C, and D, respectively, while both negative sera produced 6% fluorescent cells. Insignificant levels of fluorescent cells were detected in populations in S and G2 phase (Chart 2). The percentage of fluorescent cells in early S (3.5 hr postrelase of TdR block) ranged from 13 to 24% and decreased steadily to relatively low levels for the duration of mid-S, late S, and G2. Sampling points for sera C and D were extended to 38 hr to assess the expression of SATA as TdR-synchronized cells moved to M and G1 phase. A slight increase in the percentage of cells expressing SATA was noted as the cells progressed into M and G1 phase.

### Table 1

**Demonstration of SATA on T2 cells (initial screening)**

<table>
<thead>
<tr>
<th>Donor classification</th>
<th>Antibody to membrane-bound antigen (membrane fluorescence)</th>
<th>Antibody to intracytoplasmic antigen (intracytoplasmic fluorescence)</th>
<th>Total positive sera from each class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma</td>
<td>No. examined: 33, No. positive: 21</td>
<td>No. examined: 26, No. positive: 8</td>
<td>21</td>
</tr>
<tr>
<td>Nonsarcoma neoplasia</td>
<td>No. examined: 37, No. positive: 5</td>
<td>No. examined: 37, No. positive: 3</td>
<td>8</td>
</tr>
<tr>
<td>Blood bank donors</td>
<td>No. examined: 24, No. positive: 1</td>
<td>No. examined: 24, No. positive: 1</td>
<td>1</td>
</tr>
</tbody>
</table>

Results from the analysis on M- and G1-phase populations are presented in Chart 3. All zero-hr samples were derived from aliquots of the pooled mitotic cells that were stored at 4° in the selection process. Thus, the 30 to 35% positive fluorescent cells seen at this time represent late M and/or early G1 cells. The incidence of SATA expression increased sharply, and at approximately the mid-point of G1 phase (11.5 hr) an average of 74% of the T2 cells had detectable SATA. This value decreased steadily to an average value of 30% fluorescent cells by 27.5 hr.

Chart 4 is a composite of Charts 2 and 3. It is evident that SATA was most readily detectable in mid-G1, having risen significantly during late M and early G1, and declining steadily through late G1 into early S. Representative photomicrographs of T2 cells examined by immunofluorescence in G1, S, and G2 are seen in Figs. 1, 2, and 3. The pattern of
The purpose of this study was to examine the relationship of the expression of a human tumor antigen to the mammalian cell cycle. T2 cells, maintained free of Mycoplasma contamination, were shown to have SATA utilizing sera derived from various donors. To ensure that only SATA was detected and not a variety of other cross-reacting cellular antigens, it was of utmost importance to select a panel of antisera to study this question. Sera from patients with nonsarcomatous neoplasia and from normal blood bank donors were used to substantiate the specificity of antisarcoma antibody activity. Some such sera contained cross-reactive antibodies that were subsequently eliminated by absorption with various cell panels. The anomalous behavior of 1 control serum (blood bank donor) is similar to that observed by Bias et al. (2). Thus, a total of 20 sera (19 from sarcoma patients and 1 from a normal blood bank donor), from 94 initially screened, remained positive after repeated attempts to absorb anti-SATA activity.

SATA expression was maximal at a time point equivalent to mid-G1 (11.5 hr). After steadily increasing through early G1 to a maximum of 68 to 79% fluorescent cells, detectability of SATA decreased to approximately 25% at the beginning of S phase. The percentage of fluorescent cells detected in mid-G1 represents a 4- to 6-fold increase over values in mid to late S and early G2, and approximately 2-fold increase over values observed in asynchronous cells. The synchrony procedures utilized for T2 cells (TdR block and mitotic selection) yielded a high level of synchrony based on the LI and MI values.

The biological significance of the SATA detected on the surface of T2 cells and its relation to the mitotic cycle are obscure. SATA was not related to the ABO and Rh blood group antigen system or to the CEA produced by an established human colon carcinoma cell line (10). Tests for cross-reactive antibodies to blood-group determinants, unrelated to the ABO, Rh system in the positive sera, were also consistently negative.

The occurrence of a discrete period of maximum SATA expression suggests some type of genetic control mechanism. This phenomenon is similar to the cell cycle dependency of the production of other macromolecules. A discrete period of tyrosinase gene expression (responsible for melanin production) was shown to be related to growth kinetics by Whittaker (43). Collagen production was shown to be confined to the period of stationary phase growth (14), and Okazaki and Holtzer (22) made similar observations concerning the synthesis of myosin. Production of myosin was confined to a time period prior to the initiation of DNA synthesis, but following cell division. Others have found that the production of proteins required for S phase occurs in G1 phase (8, 36, 38). Buell and Fahey (4) observed maximum synthesis of IgG and IgM in G1 and S phase, suggesting a limited period of gene transcription. According to Boyse et al. (3), the genetic information for the production of tumor antigens is either introduced by an infectious agent (extrinsic) or is present in the cell genome before transformation occurs (intrinsic). Morton et al. (19) and Priori et al. (28) have reported the presence of virus particles in human sarcomas but these have never been seen in T2 cells by electron microscopy. Yet, it is possible that SATA depends on an oncogenic virus incorporated in the cellular genome in the lysogenic form. The synthetic mechanisms of a host cell subjected to the control of viral nucleic acids could conceivably result in the synthesis of altered products at discrete periods during the cell cycle.

Various changes in the properties of membrane components have been correlated with the position of cells in the cell cycle. Structural alterations in the cell surface of transformed Chinese hamster cells were examined with scanning electron microscopy by Porter et al. (27). The blebbed appearance of G1 cells was in marked contrast to the flattened appearance of cells in S and G2. Shoham and Sachs (32) and Smets and DeLey (33, 34) observed cyclic variations in mobility of membrane receptors. Concanavalin A receptor sites were more readily clustered in the early interphase of transformed fibroblasts and in the mitotic phase of normal
fibroblasts. Earlier, Nicolson (20) attributed a similar phenomenon to some membrane structural defect in malignant cells, possibly due to virus-induced modification. By comparison, SATA may be a structural component of the cell surface, assume the appearance of blebbed excrescences, and appear quantitatively most abundant in mid-G1. Conversely, SATA components may become mobilized and cluster during G1 in a manner analogous to Concanavalin A receptor sites in transformed cells. Such phenomena would lead to maximum detectability at discrete periods of the cell cycle.

Membrane components unrelated to the antigenic determinants may also influence their expression. The carbohydrate moiety commonly encountered at the cell surface could mask antigenic activity, and thus, fluctuations in the expression of SATA could be governed by masking and unmasking on the surface of T2 cells. The composition of the carbohydrate moiety has been shown to be dependent upon the availability of cleavage enzymes (26). Modulation of neoantigen expression has, in fact, been attributed to molecular cleavage (26). Thus, SATA may exist as carbohydrate moieties of the cell surface and be controlled by molecular effects such as enzymic cleavage which, in turn, are cell cycle dependent.

In conclusion, SATA, detectable on T2 cells by indirect immunofluorescence, has maximum expression at mid-G1. This is the first demonstration of variation of a presumably nonviral tumor antigen in synchronized human tumor cells utilizing human antitumor antibody. Biosynthetic events responsible for this phenomenon and the establishment of the chemical and physical characteristics of SATA await further study.

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REFERENCES

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Figs. 1 and 2. Indirect immunofluorescence on T2 cells synchronized in G1. Cells were incubated with positive sarcoma serum, and FITC-labeled goat anti-human IgG. × 730.

Fig. 3. Indirect immunofluorescence on T2 cells synchronized in late S and early G2. Cells were incubated with positive sarcoma serum and FITC-labeled goat anti-human IgG. All cells are clearly negative for membrane fluorescence. × 730.
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