Studies on the Antigens of Human Tumors
II. Demonstration of a Soluble Specific Antigen (G) in Cell Lines Derived from Malignant Human Tissue

JOHN M. McKENNA, RONALD P. SANDERSON, FRANK E. DAVIS, AND WILLIAM S. BLAKEMORE

Harrison Department of Surgical Research, Schools of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Summary

The presence of a soluble specific antigen (G) in cell lines derived from malignant human tissues has been demonstrated by agar gel microimmunodiffusion. The antigen was lacking in diploid cells of human origin and in hyperploid cells derived from nonneoplastic tissues with the exception of the Minnesota embryonal esophagus (MEE) cell line. The G antigen has been shown to be serologically distinct from HSL antigen, and the former's presence has been shown to correlate reasonably well with the growth potential of the cells in the hamster cheek pouch. The G antigen was not found in a number of murine cells of neoplastic and nonneoplastic origin or in monkey cell lines derived from normal tissues indicating a degree of species specificity. Only 1 of the cell lines of presumed murine origin (Ca 755) did not react specifically in agglutination tests.

Introduction

The existence of an unique soluble antigen (G antigen) found in HeLa cells and in certain human tumors has been described recently by us (38). The antigen was not discernible in a variety of nonmalignant human tissues or in benign human tumors. The absence of a predictable pattern for the distribution of G antigen in the tumors in part prompted further studies using cell lines and strains to determine the distribution of the G antigen in these cells, and perhaps to correlate its presence with some particular state of ploidy of the cells in culture. The studies to be reported in this paper have been expanded from those of an earlier report (35) to include comparisons using microimmunodiffusion in agar gel of 21 cell lines and/or strains of presumed nonmalignant human origin, 2 cell lines of presumed monkey origin and 6 cell lines of presumed murine origin. Data will also be given to show that the G antigen, partly purified by emulsification with fluorocarbon, is different in serologic reactivity from the HSL antigen, obtained by ammonium sulfate fractionation as described recently by Charney and Coriell (8).

Materials and Methods

Preparation of Antigens

The G antigen was prepared from HeLa cells as described previously (38). The cell lines and strains either were purchased as frozen pellets or grown as monolayers in Eagle's minimum essential medium supplemented with 10% calf serum. Frozen cells were homogenized at 4°C for 5 min to yield 20% suspensions in distilled water. Monolayers of cells were removed from the culture bottles with 0.25% trypsin in Hanks' solution at 34°C for 10 min. The resulting suspensions were centrifuged at 200 x g for 5 min at 4°C, and the cells lysed in 5 volumes of distilled water.

SeroLogic Assay System

The same rabbit HeLa (G) antiserum as that described in our earlier paper (38) was used in these studies. Absorption of the antiserum was carried out in the same fashion and with the same concentrate of human tissues as before (38). This human tissue concentrate was designated MT-1 and was obtained from an autopsy specimen of liver and spleen from a patient with Hodgkin's disease. Microimmunodiffusion in agar gel was accomplished as previously described (38) except that incubation was done at 4°C.

Results

The distribution of G antigen in cell lines and strains is given in Table 1 and a typical precipitin pattern is shown in Fig. 1A. Perhaps the most striking feature in these data is that the G antigen was found only in those cell lines of known malignant origin with the exception of the Minnesota embryonal esophagus (MEE) line of Syverton and McLaren (49). The G antigen was not found in the LH 344 cell line which was derived by Leonard Hayflick of the Wistar Institute from a human glioblastoma multiforme. The presence of the G antigen in cell lines, therefore, is not related to the total number of chromosomes, as all of the established lines studied show chromosome numbers in the sixties or seventies irrespective of the stated original source of the cells. Of additional interest was the observation that the HeLa G antigen was not found in any of the 6 murine or the 2 monkey cell lines, suggesting that the G antigen is indeed pe-
TABLE 1
CELL LINES AND STRAINS STUDIED

<table>
<thead>
<tr>
<th>Cell</th>
<th>Presumed species</th>
<th>Origin*</th>
<th>Malignant origin</th>
<th>G antigenb</th>
<th>Modal chromosome No.²</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa⁵</td>
<td>Human Cervix (22)</td>
<td>+</td>
<td>+</td>
<td>79 (44)</td>
<td></td>
</tr>
<tr>
<td>HEp-2⁵</td>
<td>Human Larynx (39)</td>
<td>+</td>
<td>+</td>
<td>76 (44)</td>
<td></td>
</tr>
<tr>
<td>KB ²</td>
<td>Human Nasopharynx (15)</td>
<td>+</td>
<td>+</td>
<td>77 (44)</td>
<td></td>
</tr>
<tr>
<td>Detroit 116P⁶</td>
<td>Human Pleural fluid (1)</td>
<td>+</td>
<td>+</td>
<td>75-85 (3)</td>
<td></td>
</tr>
<tr>
<td>Detroit⁵</td>
<td>Human Bone marrow (2)</td>
<td>+</td>
<td>+</td>
<td>64 (44)</td>
<td></td>
</tr>
<tr>
<td>J111¢</td>
<td>Human Blood (13, 42)</td>
<td>+</td>
<td>+</td>
<td>111 (44)</td>
<td></td>
</tr>
<tr>
<td>MAC 21¢</td>
<td>Human Lung (6)</td>
<td>+</td>
<td>+</td>
<td>79 (6)</td>
<td></td>
</tr>
<tr>
<td>LH 344¢</td>
<td>Human Glioblastoma multiforme</td>
<td>+</td>
<td>-</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Chang³</td>
<td>Human Liver (7)</td>
<td>-</td>
<td>-</td>
<td>70 (44)</td>
<td></td>
</tr>
<tr>
<td>Chang³</td>
<td>Human Conjunctiva (7)</td>
<td>-</td>
<td>-</td>
<td>72-80 (34)</td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>Human Amnion (17)</td>
<td>-</td>
<td>-</td>
<td>72-76 (43)</td>
<td></td>
</tr>
<tr>
<td>WISH⁴</td>
<td>Human Amnion (20)</td>
<td>-</td>
<td>-</td>
<td>74 and 75 (44)</td>
<td></td>
</tr>
<tr>
<td>Detroit 98¢</td>
<td>Human Bone marrow (1)</td>
<td>-</td>
<td>-</td>
<td>63 (44)</td>
<td></td>
</tr>
<tr>
<td>MEE/²</td>
<td>Human Embryonal esophagus (49)</td>
<td>-</td>
<td>-</td>
<td>67 (44)</td>
<td></td>
</tr>
<tr>
<td>Intestine 407¢</td>
<td>Human Embryonal intestine (31)</td>
<td>-</td>
<td>-</td>
<td>76 (44)</td>
<td></td>
</tr>
<tr>
<td>Girardi heart¢</td>
<td>Human Atrial appendage (24)</td>
<td>-</td>
<td>-</td>
<td>Unknown but heteroploid¢</td>
<td></td>
</tr>
<tr>
<td>WI 26⁵</td>
<td>Human Embryonal lung (30)</td>
<td>-</td>
<td>-</td>
<td>Diploid (30)</td>
<td></td>
</tr>
<tr>
<td>WI 36⁵</td>
<td>Human Embryonal lung (30)</td>
<td>-</td>
<td>-</td>
<td>Diploid (30)</td>
<td></td>
</tr>
<tr>
<td>BR 87⁵</td>
<td>Human Lung (30)</td>
<td>-</td>
<td>-</td>
<td>Diploid (30)</td>
<td></td>
</tr>
<tr>
<td>MRC 1²</td>
<td>Human Embryonal lung (30)</td>
<td>-</td>
<td>-</td>
<td>Diploid (30)</td>
<td></td>
</tr>
<tr>
<td>BSC 1²</td>
<td>Simian Kidney (30)</td>
<td>-</td>
<td>-</td>
<td>Diploid (30)</td>
<td></td>
</tr>
<tr>
<td>Salk²</td>
<td>Simian Heart (47)</td>
<td>-</td>
<td>-</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>P 1534¹</td>
<td>Murine Solid tumor transplant (14, 19)</td>
<td>-</td>
<td>-</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Ca 755²</td>
<td>Murine Breast (23)</td>
<td>+</td>
<td>-</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>L 929²</td>
<td>Murine Clone of L strain (48)</td>
<td>-</td>
<td>-</td>
<td>Unknown but heteroploidb</td>
<td></td>
</tr>
<tr>
<td>LH 420²</td>
<td>Murine Breast</td>
<td>+</td>
<td>-</td>
<td>66 (44)</td>
<td></td>
</tr>
<tr>
<td>S 180⁹</td>
<td>Murine Tumor transplant (14, 18)</td>
<td>+</td>
<td>-</td>
<td>79 (44)</td>
<td></td>
</tr>
<tr>
<td>S 91¹ (Cloudman)</td>
<td>Murine Base of tail (20)</td>
<td>+</td>
<td>-</td>
<td>Unknown</td>
<td></td>
</tr>
</tbody>
</table>

* Figures in parentheses indicate literature references.

† Plus sign (+) indicates presence of an antigen identical with HeLa G antigen using HeLa G antiserum completely absorbed for antibodies to normal human components.

‡ Purchased as frozen pellets from Microbiological Associates, Inc., Bethesda, Md.

§ As a monolayer through the courtesy of Reids Cailleau, University of California Medical Center, San Francisco, Calif.

¶ As monolayers through the courtesy of Leonard Hayflick, Wistar Institute, Philadelphia, Pa.

# As a live frozen pellet through the courtesy of Mary E. Pollock, University of Minnesota, Minneapolis, Minn.

* Anthony J. Girardi, Wistar Institute, personal communication.

** Leonard Hayflick, Wistar Institute, personal communication.

culiar to human cell lines of malignant origin and to some human tumors (38).

The nearly ubiquitous presence of pleuropneumonia-like organisms (PPLO) in stable cell lines raised the question whether G antigen might derive from these organisms. A rabbit antiserum to Type 1 PPLO (the most common contaminant) together with its homologous antiserum were provided through the courtesy of H. E. Morton of the Department of Microbiology. This antiserum, which prevented the growth of PPLO in broth cultures, gave a classical reaction of nonidentity with the G antigen-antibody system (Fig. 1B), and upon absorption failed completely to abrogate G activity (Fig. 1C). Thus no evidence was found that G antigen was in any way related to PPLO.

In an earlier paper (35), we described the presence of complement-fixing antigens extracted from 2 supposed murine cell lines (P1534 and Ca 755) which reacted with both HeLa G and J111 G rabbit antisera. Apparently this complement-fixing reactivity of these 2 cell lines was not due specifically to G antigen, as G antigen was not present in either of the cell lines when they were assayed, both before and after extraction with fluorocarbon, against absorbed HeLa G rabbit antiserum. It was felt also that perhaps these 2 cell lines were not murine as supposed, but were human cells, thus accounting for the cross-reactivity in...
complement-fixation tests. Two avenues of approach were used based on the sharing of surface antigens between somatic cells and erythrocytes of the same species. These were the hemagglutination technic of Brand and Syverton (5) and the mixed agglutination technic of Coombs et al. (10). Rabbits were immunized with 5 ml of a 10% suspension of washed human "A," ovine, and murine red cells by i.p. inoculation every other day for a total of 5 injections. Animals were bled 10 days after the last injection and the sera tested after inactivation for hemagglutinins, a 0.5% suspension of both red cells and cultured cells being used. The results, given in Table 2, indicate that with the exception of Ca 755, all the murine cells used were indeed of murine origin.

Mixed agglutination tests were performed with the same antisera. Antisera were diluted in 2-fold decrements after inactivation, and incubated with suspensions of the cultured cells. After excess antibody was washed away, suspensions of red cells were added and the test samples were examined by phase microscopy for mixed agglutination. The results, shown in Table 3, again indicate that with the exception of Ca 755, all the murine cells were of murine origin. It would appear that the Ca 755 cell line (at least as used in our experiment) was of human rather than murine origin.

Four cell lines of presumed human origin, HeLa and J111 (G-positive) and Chang conjunctiva and WISH (G-negative) were found to be of human origin by both tests.

No attempt is made to advocate the sensitivity or accuracy of one technic over the other. However, it was felt that since the hemagglutination technic of Brand and Syverton (5) used cultured cells rather than red cells as immunogens, it would not be quite fair to draw definite conclusions when we used red cells as the immunogens. The technic of Coombs et al. (10), on the other hand, prescribes the use of red cells as immunizing agents. We did the latter, and both tests gave essentially the same results.

The complete lack of identity between HeLa G and HSL antigens is demonstrated in Fig. 1D. HSL was prepared in our laboratory according to the technic of Charney and Coriell (8). Rabbits were immunized according to their procedure as well as by the procedure which we have employed using Freund's complete adjuvant (38). In our hands, at least, the rabbit antisera resulting from the use of the adjuvant gave stronger precipitin lines in microimmunodiffusion tests. However, no qualitative differences were noted between the sera resulting from the 2 immunization schedules.

### Discussion

The G antigen has been found, with 2 exceptions, only in cell lines of human malignant origin. The G antigen, as well as HSL, has been found in MEE, a cell line of nonmalignant origin, and has not been found in LH 344, a cell line derived from a glioblastoma multiforme. With regard to the latter cell line, it may be recalled that the G antigen was found in only 31% of human malignancies (35). Berman et al. (3) have suggested that, unless it is assumed that all cells in a specimen of neoplastic tissue are malignant, it is impossible to determine whether the cells selected by culture in vitro are indeed neoplastic. In addition, the isolation of apparently nonneoplastic cells from neoplastic tissue has been reported earlier (11, 23). The opposite finding with the MEE line may be more difficult to explain in the light of present evidence. The presence of G antigen in any cell or tissue of nonmalignant origin has not heretofore been encountered.

A possible correlation of G antigen with blood group antigens would have to postulate that G could be demonstrated both in (a) extracts of malignant tissues or cells of malignant origin and (b) nonmalignant tissues, diploid strains and hyperploid lines of nonmalignant origin. This does not fit the pattern of our findings. A further postulate would have it that all of the G-positive tissues or cells came from persons with the same blood group. Our previous experience with tests for G antigen in 123 malignant, 27 normal, and 10 benign human tissues (38) showed no common basis concerning the ABO blood group system, at least.

Kelus et al. (32) and Moor-Jankowski (40) demonstrated the presence of H, M, and N antigens on HeLa cells, and Moor-Jankowski (40) showed the presence of A, H, and M on KB

### Table 2

<table>
<thead>
<tr>
<th>Cultured cells (agglutinogen)</th>
<th>Human</th>
<th>Murine</th>
<th>Ovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>J111</td>
<td>40</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Chang conjunctiva</td>
<td>40</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>HeLa</td>
<td>40</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>WISH</td>
<td>20</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>P 1534</td>
<td>&lt;5</td>
<td>20</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Ca 755</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>L 929</td>
<td>&lt;5</td>
<td>40</td>
<td>&lt;5</td>
</tr>
<tr>
<td>LH 420</td>
<td>&lt;5</td>
<td>20</td>
<td>&lt;5</td>
</tr>
<tr>
<td>S 180</td>
<td>&lt;5</td>
<td>40</td>
<td>&lt;5</td>
</tr>
<tr>
<td>S 91</td>
<td>&lt;5</td>
<td>40</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Human red blood cells</td>
<td>100</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Murine red blood cells</td>
<td>&lt;5</td>
<td>100</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Ovine red blood cells</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>320</td>
</tr>
</tbody>
</table>

* Titers are expressed as the reciprocal of the dilution of antisemur showing at least 2+ agglutination of the cells.

### Table 3

<table>
<thead>
<tr>
<th>Cultured cells (agglutinogen)</th>
<th>Human</th>
<th>Murine</th>
<th>Ovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>J111</td>
<td>160</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Chang conjunctiva</td>
<td>320</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>HeLa</td>
<td>320</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>WISH</td>
<td>320</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>P 1534</td>
<td>&lt;5</td>
<td>320</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Ca 755</td>
<td>10</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>L 929</td>
<td>&lt;5</td>
<td>640</td>
<td>&lt;5</td>
</tr>
<tr>
<td>LH 420</td>
<td>&lt;5</td>
<td>160</td>
<td>&lt;5</td>
</tr>
<tr>
<td>S 180</td>
<td>&lt;5</td>
<td>160</td>
<td>&lt;5</td>
</tr>
<tr>
<td>S 91</td>
<td>&lt;5</td>
<td>320</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

* Titers are expressed as the reciprocal of the dilution of antisemur showing mixed agglutination.
cells. Hagiwara (28) found A, B, H, and M antigens on FL amnion. The absence of A and B antigens in the presence of H antigen on HeLa cells was reported (32) to agree with the information that the patient from whom the HeLa cell was derived belonged to Blood Group O. The presence of M antigen on FL cells (G-negative) precludes the connection of this antigen with G. Thus, there appears to be no correlation between the blood groups of the cells and the presence of G antigen.

A hypothesis that G antigen is related to PPLO contamination of the cells or infection of the patient where G antigen was found would have to postulate that (a) only cells of malignant origin were contaminated, (b) only patients with malignancy were infected, and (c) all were contaminated and/or infected with the same PPLO in order to establish serologic identity of the antigen. Our own evidence and the tests of Charney and Coriell (8) demonstrated quite clearly that such is not the case. Furthermore, the statistical chances of such an event would be infinitesimally small.

It was felt early in the work that the presence or absence of G antigen might be correlated with either the modal chromosome number of the cells or with a particular state of ploidy of the cells. A glance at the modal chromosome numbers of the cells studied (Table 1) will reveal that modal chromosome numbers for most of the established cell lines were in the sixties or seventies with no apparent relationship to the presence or absence of G antigen. One aspect of the report of Clausen and Syverton (9) revealed that in the stable human cells analyzed karyologically, there was a 2- to 3-fold increase in short submetacentrics and essentially the complete absence of long acrocentrics when compared with normal human male cells. Attempted karyologic analysis of HeLa, MEE (G antigen-positive cells), and WISH and WI 26 (G antigen-negative cells) kindly done by Paul S. Moorhead of the Wistar Institute were not conclusive, owing to extreme variability of the chromosomal morphology of the cells. This finding was not inconsistent with the results of Saksela et al. (46), who showed that different sera exerted a marked effect on the distribution of the chromosome numbers of HeLa cells.

Foley et al. (21) studied the heterotransplantability of 63 established cell lines by implantation of quantitated inocula into the cheek pouches of Syrian hamsters. With few exceptions, the hamster distinguished between cells of normal and neoplastic origin. Cell lines of neoplastic origin grew after implantation of similar inocula. Cell lines of embryonal origin studied were variable in their growth potential. A statistical comparison of 10 cell lines used by Foley (21) and by us (35) was made for possible correlation between these factors. A comparison coefficient of -0.76 was found. When tested for significance according to Ezekiel (16) a probability value of 0.01 was found. While it may be possible that the cell lines handled in the 2 laboratories were not the same, such a high degree of significance cannot be ignored. Other work by McKenna et al. (36), using several sublines of the KB cell line tested for both properties within 3 or 4 passages of each other, shows about the same degree of correlation.

The dissimilarity between the G and HSL antigens is intriguing from several aspects. First, it shows the presence of 2 distinct specific antigens or antigenic families in certain cell lines. One of these, HSL, apparently is associated with certain cell lines with no known predictable pattern for its distribution, but at least is unrelated to normal or malignant origin. The other antigen, G, is often found in conjunction with HSL, but is in some as yet undetermined manner apparently associated with malignancy. Second, both antigens are more often than not found in the aqueous phase after fluorocarbon emulsification of HeLa cells (see Fig. 1D and also Fig. 2 in Ref. 8), but the ammonium sulfate fractionation method (8) used for isolating HSL apparently eliminates the G antigen. Third, both antigens have remarkably similar physical and chemical characteristics. Both antigens appear to have molecular weights of about 150,000, are exceedingly heat-labile, and are soluble in 50% but insoluble in 67% saturated ammonium sulfate (8, 37). However, the G antigen has an ultraviolet spectrum characteristic of nucleoprotein in those fractions from Sephadex G-200 columns where specific serologic activity was found. The most highly purified preparations of HSL were reported to have ultraviolet spectra characteristic of protein only (8).

Perhaps the most tempting explanation to account for the appearance of G antigen in cell lines of human malignant origin and in some human tumors (38) is to postulate the activity of a cell-integrated virus genome. The appearance of new cellular antigens in polyoma virus-induced and SV40 virus-induced tumors is well established (4, 12, 25-27, 33). The antigens are specific with respect to the inducing virus. Similarly, Old et al. (41) reported that the Friend and Rauscher viruses induced the same antigenic change in the spleen cells of infected animals, and Roane and Roizman (45) reported the appearance of new cellular antigens in HEp-2 cells infected with herpes simplex virus. Thus, it could be advanced that the appearance of the G antigen in malignant cells and human tumors might be the result of some viral infection of the cells in culture and/or of the individual from whom the cells or tumor tissues were taken. In view of the ubiquitousness of viruses, this does not seem to be an unreasonable view. The exact significance of this possible sequence of events in oncogenesis remains to be established.

References

7. Chang, R. S. M. Continuous Subcultivation of Epithelial-like


FIG. 1.—A, Center well: HeLa G Ab + MT-1. Peripheral wells: 1, HeLa G; 2, J111 (aqueous extract); 3, FL amnion (aqueous extract); 4, Detroit 6 (aqueous extract). Note positive identity tests with J111 and Detroit 6.

B, Wells: 1, HeLa G; 2, PPLO Type 1 Ab; 3, HeLa G Ab; 4, PPLO Type 1. Note complete lack of identity between PPLO and HeLa preparation. HeLa G Ab was not absorbed.

C, Center well: HeLa G Ab + MT-1. Peripheral wells: 1, HeLa G; 2, HeLa G + PPLO Type 1 Ab; 3, HeLa G + PPLO Type 2 Ab; 4, HeLa G + PPLO Type T-5 Ab. Note persistence of G reaction with undiminished intensity.

D, Wells: 1, HeLa G Ab + MT-1; 2, HSL Ab + MT-1; 3, HeLa G; 4, HSL. Note presence of HSL Ab in HeLa G antiserum, but the lack of HeLa G Ab in HSL antiserum.
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