Antigens on Human Tumor Cells Assayed by Complement Fixation with Allogeneic Sera

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ABSTRACT

A well-documented report showed that human tumor cells acquired a new antigen, heterologous membrane antigen (HMAg), from heterologous serum-supplemented media. Cellular cytotoxicity and immune adherence assays that involve human tumor cells grown in fetal calf serum (FCS) have been shown to be influenced by the presence of this antigen on target cells.

This report describes the effect of HMAg on the detection of cellular antigens of human tumor cells by human serum antibodies in the complement-fixation assay. FCS and/or partially purified HMAg (P-HMAg) were used as the HMAg source. The P-HMAg was extracted from FCS by 75% ethanol. The cellular antigenic extract was prepared from human osteosarcoma (SA2) cells grown in FCS-supplemented medium by freeze-thaw and ultrasonic extraction. The cellular extract contained HMAg by the immune adherence inhibition assay.

Complement fixation detected the incidences of natural antibody to HMAg in 51 of 100 (51%) of sarcoma patients' sera and 32 of 62 (52%) of normal donors' sera (p = 0.94). Reactivity of sarcoma sera to the FCS-grown SA2 extract was much higher, 54 of 63 (86%), than in normal donors' sera, 18 of 55 (33%) (p < 0.0001). Some sera reacted to both antigens, while others reacted to P-HMAg but not to the cellular antigens, and vice versa. Quantitative absorption with insolubilized FCS or sheep erythrocytes of the serum samples that reacted to both antigens completely removed antibody activity to P-HMAg or FCS, whereas reactivity against cellular antigens remained unaffected.

Antigen extracts prepared from agamma human serum-grown SA2 cells and a biopsied liposarcoma specimen were free of HMAg. Antibody reactivity of the test sera to these antigens was essentially the same as that to the antigens prepared from FCS-grown SA2 cells. There was an almost complete reciprocity of antibody titer to these three antigenic extracts.

Therefore it may be inferred that the presence of HMAg on cultured human tumor cells does not influence the results of complement fixation.

INTRODUCTION

It has been well documented in animal models that the membrane of the normal cell is changed during neoplastic transformation. Certain new components may emerge (21, 37). Likewise, human neoplasms acquire certain components that are not present in their normal counterparts (11, 26, 34, 38). Even though the chemical and physical nature of these components is not understood, they are recognized as tumor-associated antigens. This recognition is based on immunological criteria. Many of the immunological studies depended on the use of tumor cells cultured in medium supplemented with FCS

The incorporation of antigens by cultured cells from the medium in which they are grown has been reported by several investigators (6, 17, 28). Using the IA assay, Irie et al. (22) demonstrated that human tumor cells cultured in a medium supplemented with FCS acquired a new surface antigen that is detectable by natural antibody in allogeneic sera. This antigen, designated as HMAg, originated from FCS. Different histological types of tumor cells incorporated the HMAg on their cell membranes at different levels of concentration. For example sarcomas, melanomas, and embryonic cells acquired larger quantities than did carcinomas and normal cells. However, when these same cell lines were adapted to A-yHS medium for at least 18 days, the HMAg activity disappeared (23).

Natural antibodies directed against HMAg were detected in human sera but not in animal sera. The incidence of these antibodies varied with age. Sera from children or young adults under 20 years of age had a high incidence of anti-HMAg antibodies, whereas sera from adults (>20 years of age) showed a decreasing incidence to 10 to 20% by age 70 (25).

In view of the frequent demonstration of natural antibodies to HMAg in human sera by the IA test (25) and the report that cellular reactivity against cultured tumor cells is influenced by the type of serum supplement in the culture medium (40), some earlier investigations documenting tumor-associated antigens on cultured tumor cells by CF (9, 10, 31, 32) may actually have detected antibodies to HMAg.

Our preliminary studies indicated that the reactivity between HMAg and natural antibody could be detected by CF (16). Furthermore, the antigen titers of melanoma cells by CF were not affected when the cells were transferred from FCS to human serum-supplemented medium (40). Therefore, this study was undertaken to evaluate the feasibility of the CF test for serological investigations of cellular antigens with human tumor cells cultured in heterologous serum-supplemented medium. The intent of this investigation was

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The abbreviations used are: FCS, fetal calf serum; IA, immune adherence; HMAg, heterologous membrane antigen; A-yHS, agamma human serum; CF, complement fixation; VBS, Veronal-buffered saline (pH 7.2); P-HMAg, partially purified HMAg; SRBC, sheep red blood cells.
to determine whether human tumor cells grown in FCS would influence the results of the CF test, rather than to characterize the human tumor-associated antigens.

**MATERIALS AND METHODS**

**Tissue Culture Cell Lines.** An osteosarcoma cell line (SA2) established by Morton et al. (32) and maintained in FCS was used throughout this investigation. This cell line has been used extensively by Irie et al. (22, 25). The SA2 cells were grown in FCS and also in A7HS for more than 8 weeks (23). Another sarcoma cell line, UCLA-SO-S1 (referred to as S1), derived from a biopsied leiomyosarcoma explant, was maintained in Roswell Park Memorial Institute Cultural Medium 1640 with 20% FCS, gentamicin, and Fungizone in No. 3012 plastic tissue culture flasks (Falcon Plastics, Oxnard, Calif.). A melanoma cell line UCLA-SO-M14, hereafter called M14, was established from a melanoma specimen and maintained as above. The malignant nature of the cell lines was demonstrated by their continuous growth in the cheek pouches of immunosuppressed hamsters by Dr. R. Richmond. The cell lines were regularly examined for Mycoplasma contamination. S1 and M14 cells were consistently culture negative for Mycoplasma, whereas SA2 cells grown in FCS or human serum were Mycoplasma positive by electron microscopy and culture method.

**Cellular Extracts of Tissue Culture Cells.** Antigens were extracted for the CF assay from tissue-cultured cells by the method described by Eliber and Morton (10). In brief, cells were grown as a monolayer in T-75 flasks (Falcon Plastics) to approximately 70 to 80% confluency. The medium was harvested by scraping and was washed 4 times with 40 to 50 volumes of VBS by centrifugation at 12,000 x g for 10 min. The washed cells were suspended in VBS to a density of 20% (v/v). This suspension was frozen and thawed twice, sonically extracted at low frequency, and used as the antigen source at a standardized dilution of 1:8.

FCS-grown SA2 cells were positive for HMAg, whereas A7HS-grown SA2 cells were negative by the IA inhibition assay. FCS-grown M14 cells also were positive for HMAg by immune adherence (40).

**Cellular Extracts of Biopsied Tumor Specimens.** Antigens were extracted from biopsied specimens of liposarcoma, osteosarcoma, and melanoma by the following procedure. After the connective and adipose tissues were removed, the tumor tissues were finely minced and passed through a 60 mesh screen to prepare a suspension of cells. The cells were washed 3 times with 6 volumes of 0.025 M sodium phosphate buffer supplemented with 0.145 M NaCl (pH 7.4). The washed cells were resuspended in 4 volumes of 0.025 M sodium phosphate-buffer supplemented with 0.145 M NaCl (pH 7.4), sonically extracted at low frequency, and used as a crude source of antigen.

**Source of HMAg.** FCS (Microbiological Associates, Bethesda, Md.) diluted with VBS to 1:8 was used as a source of HMAg.

HMAg also was partially purified from FCS by ethanol precipitation. Three parts of absolute ethanol were mixed with 1 part of the serum. The ethanol was added drop by drop with continuous stirring at room temperature. After the mixture was stirred for 2 hr at room temperature and then centrifuged at 800 x g for 10 min. The supernatant was dialyzed against a large amount of distilled water for 3 days at 4°. The dialyzed fraction was concentrated to the original volume of the FCS by vacuum dialysis. The concentrate was further dialyzed overnight against 4 liters of VBS and then used as P-HMAg. Analysis of the P-HMAg revealed that on an equal-volume basis it had approximately 0.001 protein concentration as compared to FCS but was significantly antigenic by immune adherence.

**Protein Determination.** Protein concentrations were determined by the Lowry et al. method (29).

**Test Sera.** Sarcoma and melanoma sera were obtained from patients of the Division of Surgical Oncology. Ages of the sarcoma patients ranged from 20 to 60 years. Control normal sera were obtained from apparently healthy laboratory personnel and UCLA medical students who had no history of malignant disease. The normal donors' ages ranged from 20 to 50 years. After collection sera were stored at −35° without any preservative and then heat inactivated at 56° for 30 min prior to use in the CF test.

**CF Test.** The presence of antigen or antibody in test samples was assessed by the ultramicro-CF technique described elsewhere (14). Essentially, the procedures were the same as those of the conventional micro-CF assay developed by Sever (39) for serological investigation of viruses and adapted by Eliber and Morton (9, 10) for tumor-antitumor systems, except that the total volume per well was 8 μl instead of 25 μl. The tests were performed in Terasaki tissue culture plates (Falcon Plastics). All wells of the plate were filled with mineral oil of light density, and the reagents, 2 μl antigen dilution, 2 μl antibody dilution, and 2 μl of complement representing 2 units, were injected into appropriate wells through the layer of mineral oil with a fixed-needle Hamilton syringe (Hamilton, Reno, Nev.) equipped with a repeating dispenser. Fresh human umbilical cord serum was used as a source of complement (8). The contents of the wells were mixed by shaking the plate on a micromixer (Cook Laboratory Products, Alexandria, Va.) for 30 sec. After incubation at 37° for 1 hr, 2 μl of 0.5% sensitized SRBC (Flow Laboratories, Inc., Rockville, Md.) were added as the indicator system. Cells from the same sheep and 1 lot of hemolysin (Flow Laboratories, Inc.), were used throughout this investigation. After another period of incubation for 30 min at 37°, the plates were read visually.

Each test plate was controlled internally as well as externally. The internal controls included a complement titration, a buffer control, a control for SRBC representing 100, 50, and 0% hemolysis, and controls for anticomplementary activity of the test sera and the antigen preparations. The external controls were maintained with a cancer patient's serum known to be positive and a normal serum known to be negative against an antigen of known antigenicity. These controls provided a close check on day-to-day variations in the titers, and when the variations were greater than 2-fold dilution either way the experiments were considered invalid.

**Definition of Titer and Positive Activity.** A titer was defined as the highest dilution of the test serum showing 50% or less hemolysis of the SRBC. A serum was consid-
erected positive when its titer was 1:4 or higher. If a test serum was anticomplementary, it was considered positive only when its titer was at least 4-fold above the highest anticomplementary dilution. Thus, a test serum with anticomplementary activity at a dilution of 1:4 and with reactivity at a dilution of 1:8 was considered negative against the target antigen by these criteria. A test serum with anticomplementary activity at 1:4 and with reactivity at 1:16 dilutions was considered positive against the target antigen, and the titer of the serum was said to be 1:16.

**IA Assay.** Detection of HMAg in cellular extracts of tumor cells was performed by the IA inhibition assay as described elsewhere (22, 23).

**Absorption of Sera.** Since SRBC contain HMAg (23) they were used as a source of HMAg for the absorption of natural antibodies to HMAg from selected sera. SRBC aged for 8 days at 4° and washed 5 times with VBS were used. Aliquots (200 µl) of the heat-inactivated (56° for 30 min) sera that were known to contain anti-HMAg antibodies by IA and CF with FCS or P-HMAg as the target antigen were mixed thoroughly with varying numbers (4 x 10⁸, 2 x 10⁷, 2 x 10⁶, and 1 x 10⁵) of the SRBC. Absorption was carried out by allowing the mixtures to incubate at 37° for 60 min with intermittent shaking and to stand overnight at 4°. The sera then were recovered by centrifugation at 5000 x g for 15 min.

In addition to SRBC, FCS insolubilized by cross-linking with glutaraldehyde (1) also was used to remove anti-HMAg antibodies. Five hundred µl of the test sera were mixed with an equal volume of immobilized FCS and incubated at 37° for 60 min. The serum samples were recovered by centrifugation at 7000 x g for 15 min. Each serum sample was subjected to 3 such successive absorptions.

Tumor cells (SA2 and M14) grown in AyHS served as the HMAg-free source for absorption of antibodies to cellular antigens. The cells were grown in Roswell Park Memorial Institute Medium 1640 (Microbiological Associates) and were supplemented with 20% AyHS (Microbiological Associates) in T-75 flasks (Falcon Plastics). The cells were harvested by scraping when growth reached about 80% confluency and were washed 3 times with approximately 40 volumes of VBS each time. Absorption was carried out by mixing 250 µl of a heat-inactivated serum sample with 1 x 10⁸ washed cells. The mixture was incubated at 37° for 1 hr and centrifuged at 3000 x g for 10 min to recover the serum samples.

**Statistical Analysis.** Significance of titers and incidence of positive reactivity against various antigen preparations were analyzed by Student's t test and χ² method with a Wang calculator. Series 500 to 600. The p values were derived from tables of normal distribution curves.

**RESULTS**

**Detection of HMAg in Cellular Extract of FCS-Grown SA2 Cells by IA Inhibition.** The presence of HMAg in FCS, P-HMAg, and cellular extracts was determined by the ability of the test material to inhibit the reactivity between a standardized dilution of anti-HMAg antibody and FCS-grown SA2 cells by 50% (22, 23). Results in Table 1 show that the highest dilution of the cellular extract from FCS-grown SA2 cells was one-eighth that of FCS and one-fourth that of P-HMAg. No detectable inhibition of IA reactivity was observed by undiluted cellular extracts of AyHS-grown SA2 cells.

**Reactivity of HMAg with Sarcoma and Normal Sera by CF.** Experiments were designed to determine whether serological reactivity between HMAg and its natural antibody could be detected by CF. Selection of sarcoma and normal sera was made on the basis of IA results (23). Three anti-HMAg-positive and 1 negative sera were taken from each of the above categories (sarcoma and normal). These sera were tested against FCS and P-HMAg. Results presented in Table 2 show that the sera that were known to contain natural antibodies to HMAg by IA also had detectable titers against FCS and P-HMAg by CF. The anti-HMAg antibody titers ranged from 1:16 to 1:64 in both the sarcoma and normal serum samples. The sera that were negative for anti-HMAg antibodies by IA did not show any reactivity by CF at the lowest serum dilution, 1:4, used in the CF assay. Lower dilutions were generally accompanied by anticomplementary activity. For confirmation of this observation, 45 randomly selected sarcoma and 12 normal sera were tested for the presence or absence of anti-HMAg antibodies in CF and IA with P-HMAg as the target. Twenty-four of the sarcoma sera were positive for the presence of anti-HMAg antibodies by CF, and 25 were positive by IA. Twenty of these sera were positive in both assays, whereas 4 were positive in CF only with antibody titers of 1:4, and 5 were positive in IA only with 50% IA titers of 1:10. Statistical analysis by χ² revealed no significant difference in the incidence of positive activity for anti-HMAg antibody in sarcoma sera by the
2 assays ($p = 0.818$). Of 12 normal sera, 7 were positive against HMAg by CF, and 6 were positive by IA. All 6 sera that were positive by IA were also positive by CF against P-HMAg. The incidence of anti-HMAg antibodies in normal sera by CF and IA was not significantly different ($p = 0.6818$).

### Comparison of Reactivity of Sarcoma Sera Against FCS-Grown and A\textsubscript{Y}HS-Grown SA2 Cell Preparations by CF

In this series of experiments, SA2 cells were grown in FCS or in A\textsubscript{Y}HS for more than 8 weeks. By IA the FCS-grown SA2 cell preparation was positive for HMAg, whereas the A\textsubscript{Y}HS-grown SA2 cell preparation was HMAg-negative. With these 2 cellular extracts as target antigen(s), 5 sarcoma sera were assayed for complement-fixing antibody titers. Table 3 shows that there were no differences in the antibody titers regardless of whether the target antigen was HMAg positive or negative by IA. The first 3 sera contained detectable natural antibodies to HMAg by CF against FCS and IA.

### Incidence of Natural Antibody to HMAg in Sarcoma and Normal Sera by CF

Results presented in Table 4 show that 51 of 100 sarcoma (51%) and 32 of 62 normal donors (52%) sera contained complement-fixing antibodies to P-HMAg. A serum was considered positive when it showed greater than 50% inhibition of hemolysis of sheep cells at a dilution of 1:4 or higher. Similar results were observed when FCS diluted 1:8 was used as a target antigen (data not shown).

### Table 3

<table>
<thead>
<tr>
<th>Test Serum</th>
<th>FCS (HMAg-positive)</th>
<th>A\textsubscript{Y}HS (HMAg-negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma\textsuperscript{a}</td>
<td>1\textsuperscript{a}</td>
<td>1:8</td>
</tr>
<tr>
<td>2\textsuperscript{b}</td>
<td>1:8</td>
<td>1:8</td>
</tr>
<tr>
<td>4\textsuperscript{b}</td>
<td>1:16</td>
<td>1:16</td>
</tr>
<tr>
<td>3</td>
<td>1:16</td>
<td>1:16</td>
</tr>
<tr>
<td>5</td>
<td>1:16</td>
<td>1:16</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Sera 1 to 4 are the same as in Table 2.

\textsuperscript{b} These sera were known to contain anti-HMAg antibodies up to titers of 1:32 against P-HMAg or FCS.

### Table 4

<table>
<thead>
<tr>
<th>Type of serum</th>
<th>No. tested</th>
<th>No. positive\textsuperscript{b}</th>
<th>% positive</th>
<th>Range of antibody titer for positive sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma</td>
<td>100</td>
<td>51</td>
<td>51\textsuperscript{c}</td>
<td>1:4-1:128\textsuperscript{d}</td>
</tr>
<tr>
<td>Normal</td>
<td>62</td>
<td>32</td>
<td>52\textsuperscript{c}</td>
<td>1:4-1:128\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} HMAg, partially purified from FCS by ethanol precipitation, was used at a dilution of 1:8 to determine the anti-HMAg activity of the test sera.

\textsuperscript{b} A serum was considered positive when it reacted at a dilution of 1:4 or higher.

\textsuperscript{c} Percentages of positivity of sarcoma and normal sera are not significantly different ($p > 0.94$) by $\chi^2$ test.

\textsuperscript{d} The mean titers were not significantly different by Student's $t$ test ($p > 0.3$).

Statistically, the percentages of reactivity of sarcoma and normal sera were not significantly different ($p > 0.94$).

### Distribution of Positive Seroreactivity of Sarcoma and Normal Sera to SA2 and Liposarcoma Extracts

Sarcoma and normal sera were screened against 3 target antigens: (a) FCS-grown SA2; (b) A\textsubscript{Y}HS-grown SA2; and (c) biopsied liposarcoma extracts. The distribution of positive reactivity against these preparations is shown in Table 5. The sarcoma sera reacted with the 3 extracts at a much higher incidence (82 to 86%) than did normal sera (31 to 34%). These differences were statistically significant ($p < 0.0001$). Furthermore, the incidence of sarcoma sera reactivity against extracts of FCS-grown SA2 (HMAg-positive), A\textsubscript{Y}HS-grown SA2 (HMAg-negative), and biopsied liposarcoma (HMAg-negative) were 86, 82, and 84%, respectively ($p > 0.5$ for any combination). Likewise, the incidence of normal sera reactivity to the above preparations was 33, 31, and 34%, respectively ($p > 0.7$ for any combination). Therefore, the FCS in tissue culture medium did not appear to influence the positive seroreactivity of sarcoma and normal sera as determined by CF.

### Absorption Studies

Sarcoma and melanoma sera were quantitatively absorbed with glutaraldehyde-insolubilized FCS and SRBC to remove natural antibodies to HMAg. Both unabsorbed and absorbed sera were reacted against tumor cell extracts, FCS, and/or P-HMAg.

Chart 1 shows that the reactivity of the 2 sarcoma sera against FCS or P-HMAg was completely abolished after absorption with $1 \times 10^6$ SRBC. The absorbed sera were still reactive to the same extent as the unabsorbed sera against extracts of FCS-grown SA2 and liposarcoma. Similar results were observed when sarcoma sera from 2 other patients were absorbed with glutaraldehyde-insolubilized FCS (Chart 2). In this experiment extracts of S1 and a biopsied osteosarcoma were used as target antigens in addition to FCS-grown SA2 and FCS.

Since HMAg has been found in melanoma cells (22), selected melanoma sera also were absorbed with the glutaraldehyde-insolubilized FCS and SRBC. The results are illustrated in Charts 3 and 4. In this experiment the target antigens were extracts of a biopsied melanoma and a melanoma cell line, M14. The melanoma sera contained significant levels of complement-fixing natural antibodies to HMAg. Quantitative absorption of the sera with FCS or SRBC completely removed the antibodies to HMAg. However, the extent of reactivity of these sera against melanoma cell extracts was not affected by the absorptions.

If the positive reactivity of sarcoma and melanoma sera to respective target antigens was due to the presence of HMAg in the FCS-grown cell preparations, a significant decrease in antibody titer should have been observed after absorption of the sera with FCS or SRBC. A 2-fold fluctuation in titers could have been due to experimental variations or slight dilution of the sera during absorptions.

Absorption of 250-\mu l aliquots of sarcoma sera with $1 \times 10^6$ A\textsubscript{Y}HS-grown SA2 cells and 250-\mu l aliquots of melanoma sera with $1 \times 10^6$ chemically defined medium-grown M14 cells (4) reduced complement-fixing antibody titers significantly against the corresponding tumor cell preparations. The extent of reactivity against HMAg was not affected (Tables 6 and 7).
Table 5
Distribution of positive reactivity of sarcoma and normal sera against sarcoma cell extracts by CF

<table>
<thead>
<tr>
<th>Source of target antigen</th>
<th>Presence of HMAg in target antigen</th>
<th>No. positive/no. tested</th>
<th>% positive</th>
<th>No. positive/no. tested</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured tumor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA2 grown in 20% FCS</td>
<td>+</td>
<td>54/63(d,d)</td>
<td>86</td>
<td>18/55(c)</td>
<td>33</td>
</tr>
<tr>
<td>SA2 grown in 20% AyHS</td>
<td>-</td>
<td>82/100(d,d)</td>
<td>82</td>
<td>18/59(c)</td>
<td>31</td>
</tr>
<tr>
<td>Biopsied tumor cells liposarcoma</td>
<td>-</td>
<td>38/45(d)</td>
<td>84</td>
<td>17/50(k)</td>
<td>34</td>
</tr>
</tbody>
</table>

\(a\) +, present; - , absent, by IA.

\(b\) A serum was considered positive when it reacted at a dilution of 1:4 or higher.

\(c\) Incidences of positive reactivity between sarcoma and normal sera were statistically significant by \(\chi^2\) test (\(p < 0.01\)).

\(d\) Incidences of positive reactivity of sarcoma sera against various target antigens were not significant by \(\chi^2\) test (\(p > 0.5\)) in any combination.

\(e\) Incidences of positive reactivity of normal sera against various target antigens were not significant by \(\chi^2\) test (\(p > 0.7\)) in any combination.

DISCUSSION

Although the chemical nature and biological significance of the HMAg is not yet known, its presence on human tumor cells grown in FCS-enriched tissue culture medium could influence those immunological investigations that use this tissue culture technique (23). It already has been demonstrated that lymphoid cell-mediated cytotoxicity and serum-ariming and serum-blocking activities are influenced by the type of serum supplement in the culture medium (40).

Though the reactivity between natural antibodies in human sera and P-HMAg or FCS can be detected by CF (Table 2), no differences in antibody titers were observed when extracts of FCS-grown SA2 (positive for HMAg by IA; Table 1) or AyHS-grown SA2 were used as target antigens (Table 3). Thus the reactivity was not influenced by the presence of HMAg on tissue culture cells (SA2) even when at least 3 of the test sera contained natural antibodies to the HMAg. If influence had been exerted, the reactivity of the first 3 sera of Table 3 would have been higher with the HMAg-positive SA2 preparation than with the HMAg-negative SA2 preparation.

Table 2 and the absorption studies show that partial purification of HMAg by ethanol precipitation of FCS preserved most of the antigenic components of HMAg. The extent of reactivity of all anti-HMAg-positive sera against FCS or P-HMAg was almost the same (Table 1). Further-
more, sera absorbed with glutaraldehyde-insolubilized FCS showed a parallel loss of reactivity against FCS and P-HMAg (Charts 2 and 4). Therefore, the use of P-HMAg in place of whole FCS was justifiable in the CF assay.

The incidence of natural antibody to HMAg in sarcoma (52%) and normal (51%) sera was almost equal (Table 4). On the other hand, as noted in Table 5, the incidence of positive reactivity of sarcoma sera to cellular extracts of FCS-grown SA2, AyHS-grown SA2, and biopsied liposarcoma was much higher (82 to 86%) than was the normal sera (31 to 33%). If the FCS in tissue culture medium had influenced the result, then the incidence of positive reactivity of normal sera against cellular extracts of FCS-grown SA2 (HMAg-positive) should have been about 50% instead of 33%.

During the analyses of incidence of reactions of sarcoma and normal sera against HMAg and tumor cell preparations (Tables 4 and 5), 4 situations were noticed and are shown in Table 8 by representative sera: Situation a, Sera D and E had significant titers (1:16 and 1:64) against HMAg but were negative against extracts of FCS-grown SA2; Situation b, Sera B and G were positive to both target antigens with titers ranging between 1:16 and 1:32; Situation c, Sera A and H had high titers (1:32 and 1:64) against HMAg but low titers (1:4) against FCS-grown SA2 extract; Situation d, Sera C and F were positive against FCS-grown SA2 extract with complement-fixing titers of 1:16 and 1:8, respectively, but were negative against HMAg. The titers of the sera against the FCS-grown SA2 cell extract were comparable with the titers against the AyHS-grown SA2 cell extract or biopsied liposarcoma extract. Under similar experimental conditions Situation a is a contrast to Situation d. Situation a would be possible if the sera had antibodies limited to HMAg. Similarly, Situation d might occur if the sera had antibodies to antigenic components of FCS-grown SA2 extract only. Because the FCS-grown SA2 cell preparation was positive for HMAg by IA and Sera D and E had natural antibodies to HMAg by CF and by IA, a positive reactivity between these sera and FCS-grown SA2 extract should have been observed. However, this was not the case even after repeated CF testing.

Situation b might have occurred for the following reasons: (a) heterogeneity of antibody in the sera, i.e., the presence of antibodies of different specificities; (b) detection of HMAg on the FCS-grown SA2 cell extract; or (c) a combination of the 2. The latter 2 possibilities could easily be eliminated by absorption studies. Our studies indicate that quantitative absorption of the natural anti-HMAg antibody-positive sarcoma and melanoma sera with insolubilized FCS or SRBC completely abolished their activity against FCS and P-HMAg. This treatment did not affect complement-fixing titers of the sera against corresponding tumor cell extracts. Both FCS-grown and AyHS-grown cell
of the non-HM antigens; rather the purpose was to deter-
mine whether the use of HMAg-positive tumor cells could
among laboratory personnel than was drawn from personnel in the Division of Oncology. We
diluted cell preparation, whereas in this study reactivity was
antibody-positive when a 1:8 dilution reacted with the
following: (a) in earlier reports (9, 30), sera were considered
significantly higher than reported earlier (31 to 33% versus
sera to extracts of SA2 and biopsied liposarcoma was
antigens by cultured cells from growth medium (6, 17, 28)
ences in reactivity, it would seem that incorporation of new
studies or absorption studies showed no significant differ-
which showed that SRBC possess HMAg (23).
preparations were included as the target antigens (Charts 1
to 4). These results confirm those of the previous report
which showed that SRBC possess HMAg (23).
Since the parallel use of antigens prepared from biopsied
liosarcoma, osteosarcoma, and melanoma in distribution
studies or absorption studies showed no significant differ-
ences in reactivity, it would seem that incorporation of new
antigens by cultured cells from growth medium (6, 17, 28)
has very little if any effect on seroreactivity seen by CF.
In this study the incidence of positive activity in normal
sera to extracts of SA2 and biopsied liposarcoma was
significantly higher than reported earlier (31 to 33% versus
18 to 20%) (9, 30). Possible explanations may include the following: (a) in earlier reports (9, 30), sera were considered
antibody-positive when a 1:8 dilution reacted with the
diluted cell preparation, whereas in this study reactivity was
considered positive when the sera reacted at a dilution of
1:4; (b) one-third of the group of normal sera in this study
was drawn from personnel in the Division of Oncology. We
have consistently noted a higher incidence of seroreactivity
to tumor cell preparations among laboratory personnel than
among nonlaboratory personnel. A similar observation has
been reported by Graham-Pole et al. (12) in a neuroblas-
toma study and by Byers et al. (3).
In this study we did not attempt to define the specificity
of the non-HM antigens; rather the purpose was to deter-
mine whether the use of HMAg-positive tumor cells could
influence the CF results. The seroreactivity observed here
against non-HM antigens might be attributed to several
antigens including the oncofetal antigen described by Irie
et al. (24), S1 and S2 antigens reported by Hirshau et al. (20)
and Mukherji et al. (35), sarcoma-associated antigen (9, 10,
30), and common cancer-associated antigen(s) (13, 15).
At present there is no clear explanation for the failure of the
CF test to detect reactivity between HMAg-positive (by
IA) tumor cell extracts and the sera that were positive for
antibodies to HMAg. It may be that the dilution of FCS-
grown tumor cell preparations used in this investigation
contained HMAg in concentrations that were too low to be
detected by CF. Therefore its presence did not interfere
with the determination of antibodies to non-HM antigens.
Regardless of the explanation results of this study indicate
that the CF assay may be useful for the detection of
antibodies to tumor-associated or other antigens when the
source of antigen is FCS-cultured cells.
Three workable methods to exclude HMAg from experi-
mmental systems that use FCS-cultured human tumor cells
have been reported by Irie et al. (23). The methods include
changing medium supplement from FCS to whole human
serum or A-7HS, inactivation or removal of HMAg by enzyme
treatment, or absorption of sera with HMAg-bearing cells.
Irie et al. (23) noted that whole human serum may cause
antigenic modulation and nonspecific immune reactions by
the natural antibodies present in the human serum. A-7HS is
expensive and one must wait 2 to 3 weeks for the HMAg to
disappear. Treatment of the cells with enzymes may destroy
or modify the antigens of interest. Absorption of sera with
HMAg-bearing cells may become impractical when a large
number of sera are to be screened. Under those circumstances
CF may be the serological assay of choice.

Table 7

<table>
<thead>
<tr>
<th>Serum</th>
<th>Treatment</th>
<th>Chemically defined medium-grown M14</th>
<th>Biopsied melanoma</th>
<th>FCS</th>
<th>P-HMAg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma 1</td>
<td>Unabsorbed</td>
<td>1:16</td>
<td>1:64</td>
<td>1:32</td>
<td>1:32</td>
</tr>
<tr>
<td>Melanoma 2</td>
<td>Unabsorbed</td>
<td>1:8</td>
<td>1:32</td>
<td>1:16</td>
<td>1:16</td>
</tr>
</tbody>
</table>

Table 8

Comparison of serological reactivity of sarcoma and normal sera against cellular extract of FCS-grown SA2 and P-HMAg by CF

<table>
<thead>
<tr>
<th>Serum</th>
<th>FCS-grown SA2 (HMAg-positive by immune adherence)</th>
<th>P-HMAg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma</td>
<td>1:4</td>
<td>1:32</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>1:16</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td></td>
<td>&lt;1:4</td>
<td>1:16</td>
</tr>
<tr>
<td>Normal</td>
<td>1:8</td>
<td>&lt;1:4</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>1:32</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>1:64</td>
</tr>
</tbody>
</table>

"A serum was considered positive if it reacted at a dilution of 1:4 or higher.

REFERENCES

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Rishab K. Gupta, Reiko F. Irie and Donald L. Morton


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